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The Impact of Bile and Anaerobic Stress on *Listeria monocytogenes* Isolates

Thesis presented by

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for the degree of

Master of Science

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School of Microbiology

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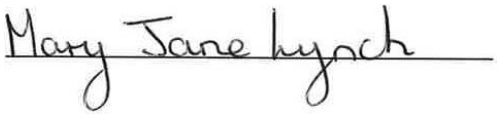
Table 2. Anaerobic Bile Resistance Assay Results

Table 3. Aerobic Bile Resistance Assay Results

Table 4. Bile Salt Hydrolase Agar Plate Results

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.



Mary Jane Lynch

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Abstract

Listeria monocytogenes is regarded as an important food pathogen that can cause serious illness. All individuals are susceptible to *L. monocytogenes* infection with pregnant women, immunocompromised people and the elderly particularly at high risk. *L. monocytogenes* infection can cause febrile gastroenteritis and a more serious invasive form where the bacteria cross the blood/brain barrier or the blood/foetal barrier.

In this study the effect of bile and anaerobic stress on *L. monocytogenes* was assessed. Bile has antimicrobial properties and it is one of the stressors that *L. monocytogenes* must overcome in the human gastrointestinal tract to establish infection. *L. monocytogenes* uses an enzyme, bile salt hydrolase (BSH), to detoxify bile. The bile resistance response may be triggered by anaerobic conditions. In total 154 food/environmental isolates and 74 clinical isolates were screened. A bile tolerance assay and a BSH enzymatic agar plate assay were developed. Experiments were carried out at a lowered pH and/or anaerobic conditions to simulate conditions in the human gastrointestinal tract where *L. monocytogenes* is exposed to bile.

Results indicate that there are bile tolerance variations in *L. monocytogenes* strains from food, environmental and clinical sources. In this large-scale study, cultures grown anaerobically were less tolerant to bile than cultures grown aerobically and generated more varied resistance patterns. The effect of anaerobiosis is not as well understood as aerobic and results provide an insight into the influence of environmental factors. Interestingly, the bile resistance assay revealed that the clinical isolates were significantly more tolerant to bile than the food and environmental isolates ($P < 0.001$). This important finding demonstrates the evolutionary pressure on these strains to survive in the gut. Correlation of results from the BSH agar plate assay with the bile tolerance assay demonstrated that the food and environmental isolates had slightly higher levels of BSH activity than the clinical isolates. However, the difference between the groups was not as significant as the bile resistance results. Results were also analysed on the level of lineage and clonal complex to identify possible links to the bile response. The results generated

from this study provide insight into important associations between bile tolerance of individual strains/lineages and efficacy of *L. monocytogenes* infection.

Literature Review

Listeria monocytogenes

L. monocytogenes is a Gram-positive facultative anaerobic bacillus bacterium. The bacterium is ubiquitous in the environment (soil, water etc) and its natural habitat is decaying vegetation. Many animals carry *L. monocytogenes* in their faeces with rates reported at 21.3% in cattle and 1.5% in sheep (Esteban *et al*., 2009). *L. monocytogenes* is classified as a foodborne pathogen and it can cause two forms of illness. The first is a non-invasive febrile gastroenteritis causing symptoms such as fever, watery diarrhoea, nausea, headache, joint and muscle aches and pains (Ooi *et al*., 2005). The more serious form is an invasive infection where the bacteria can cross the blood brain barrier and/or the blood foetus barrier causing symptoms such as meningitis, sepsis, abortion and still birth. The infection rate is low but there is a high mortality rate, as high as 20 to 30%. All individuals are susceptible to listeriosis with pregnant women, the elderly and immunocompromised most at risk of infection. Listeriosis is a notifiable disease in Ireland. The most recent statistics in Ireland, from 2020, available from the Health Protection Surveillance Centre, identified 6 cases of listeriosis. This was a decrease from previous years, most likely due to the impact of the Covid 19 pandemic.

The majority of *L. monocytogenes* infection is as a result of eating contaminated foods. Ready-to-eat foods are commonly implicated as there is no further heat or processing step to reduce the numbers of the bacteria. Typical foods associated with *L. monocytogenes* infection include coleslaw, raw milk, ice cream and soft cheeses. Non-typical foods include celery, cantaloupe, mung bean sprouts, stone fruits and caramel apples (Buchanan *et al*., 2017). The bacterium is noted for its persistence in food processing environments. Leong *et al*., 2014 monitored 48 food processing facilities from 2013 to 2014, of which 43 produced ready-to-eat foods. 62% of the facilities were positive for *L. monocytogenes* over the course of the study. Isolates were characterised by serotyping and pulsed field gel electrophoresis (PFGE). Seven

facilities were positive for persistent strains with evidence of cross contamination from the environment to food in four of the facilities. A study completed by Carpentier *et al*., 2011 into the persistence of *L. monocytogenes* in food processing facilities identified factors affecting persistence including adhesion potential, biofilm formation, resistance to desiccation, acid, heat and tolerance of disinfectants. Favourable growth conditions include harbourage sites such as floors, drains, food processing equipment and the wet environment of refrigerated rooms. Heir *et al*., 2004 investigated if persistent strains had more resistance mechanisms to aid survival. The study concluded that persistent strains were not more resistant to disinfectants and did not have a higher resistance to desiccation. The presence of *L. monocytogenes* highly correlated to surfaces being soiled, a neutral or alkaline pH, a relative humidity higher than 70% and temperatures lower than 10 °C. The bacterium is quite hardy. It is salt tolerant, at levels as high as 10% and one of its most noted survival characteristics is its ability to grow and survive at low temperature (as low as 1 °C).

EU legislation, EC Number 2073/2005 Microbiological Criteria for Foods, divides foods into two groups, those that cannot support the growth of *L. monocytogenes* and those foods that can support growth. In the absence of evidence, it is assumed that the food supports growth. The regulation categorises all *L. monocytogenes* strains as equally virulent. For foods that can support the growth of *L. monocytogenes*, EU legislation denotes the absence of *L. monocytogenes* in 25g (number of samples =5) before the food has left the food producer (EN/ISO 11290-1) and a limit of 100 cfu/g (EN/ISO 11290-2) during the shelf life of the product on the market. Foods that cannot support growth have a limit of 100 cfu/g (EN/ISO 11290-2) during the shelf life of the product on the market.

L. monocytogenes was first identified in 1924 by E.G.D. Murray in England after the sudden deaths of six rabbits followed by isolation of small Gram-positive bacilli from rabbits and guinea pigs (Murray *et al*., 1926). The bacterium was first called *Bacterium monocytogenes* due to the production of a large mononuclear leucocytosis. *L. monocytogenes* EGDe (Lineage II, serotype 1/2a) was the first strain to be isolated and has become a model Listeria strain. *L. monocytogenes* was not

identified as a food pathogen until 1981 (Schlech *et al*., 1983). There have been numerous high-profile outbreaks caused by *L. monocytogenes* to date. In the most recent European Union One Health Report (2021), the European Food Safety Authority (EFSA) recorded 2183 cases of reported Listeriosis in the EU in 2021 with 923 hospitalisations and 196 deaths. Listeriosis was the fifth most common zoonotic infection in the EU.

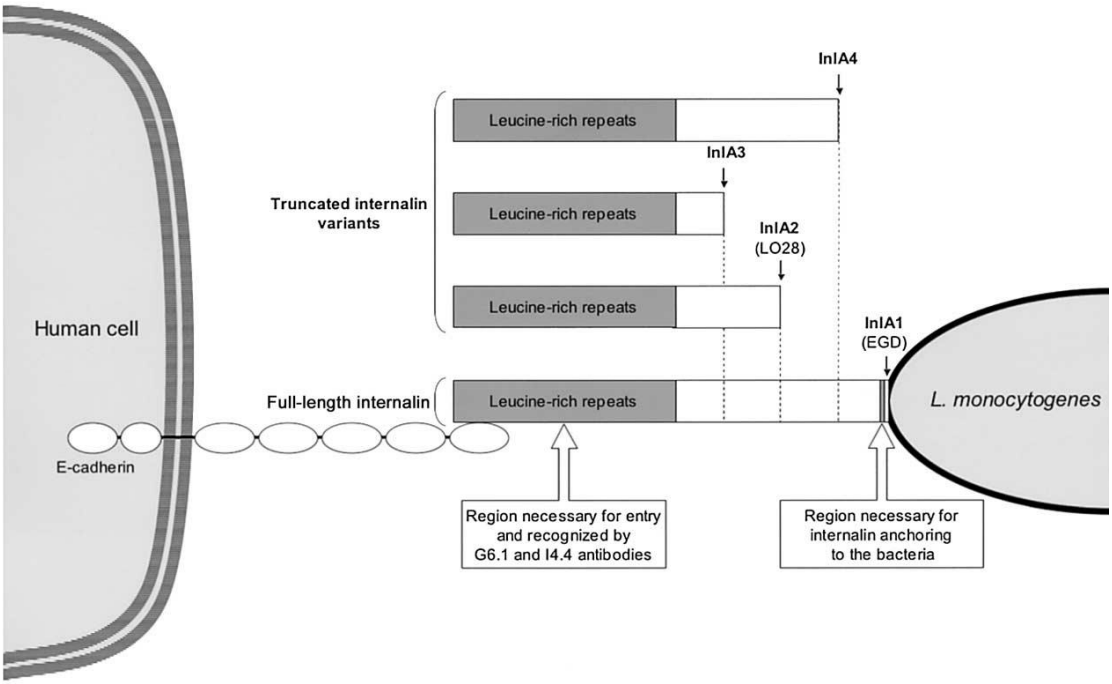
There are 14 *L. monocytogenes* serotypes identified to date (with newly identified hypervirulent serovar 4h), separated into four different evolutionary lineages (I, II, II, IV) (Lakicevic *et al*., 2023). Lineage I and II are predominantly associated with human clinical cases. More specifically, Lineage I is typically associated with clinical cases and Lineage II strains can be found in food and the environment in addition to animal listeriosis cases and sporadic human cases (Orsi *et al*., 2011). Lineage III and IV are relatively rare and are mainly isolated from animal sources (Orsi *et al*., 2011). Lineage I and II account for 9 of the known serotypes: 1/2b, 3b, 4b, 4d and 4e in Lineage I and 1/2a, 1/2c, 3a and 3c in Lineage II. Lineage I serotype 4d and 4e are rare in food and clinical samples but are highly similar to serotype 4b (Lomonaco *et al*., 2015). Lineage III contains serotypes 4a, 4c and some strains belong to 4b. Serotypes 1/2b and 4b from Lineage I and serotype 1/2a from Lineage II are most commonly isolated from clinical cases. Serotypes 1/2a, 1/2b, 1/2c and 4b account for 98% of the strains isolated from clinical cases (Jacquet *et al*., 2002). Approximately 50% of the core genes in *L. monocytogenes* display evidence of recombination with Lineage II having a higher genetic diversity. Lineage I has been shown to be less prone to recombination (Lomonaco *et al*., 2015).

Recent advances in epidemiology have identified Whole Genome Sequencing (WGS), a next generation sequencing technology, as the gold standard for outbreak investigations and public health investigations (Lakicevic *et al*., 2023). WGS identifies the entire (or near entire) DNA sequence of a microorganism's genome. PFGE is traditionally used to type *L. monocytogenes* strains and to trace outbreaks but Public Health Laboratories are moving away from PFGE based methods to WGS based typing. Multi-Locus Sequence Typing (MLST) is another commonly used method to characterise *L. monocytogenes* isolates using seven housekeeping genes to

determine the allelic profile or sequence type (ST). WGS data can be used to study genetic relatedness between strains including single-nucleotide polymorphisms (SNP), gene-by-gene allelic profiling using the core genome multi-locus sequence typing (cgMLST) or whole genome multi-locus sequence typing (wgMLST) (Lakicevic *et al*., 2023). From a *L. monocytogenes* perspective, WGS can be used to track global epidemiology and study virulence, evolution and population diversity (Buchanan *et al*., 2017). The advantages of a WGS approach include the speed of analysis, it provides a high-resolution view of the genome and has superior discriminatory power. Disadvantages include the cost and requirement for appropriate IT infrastructure. Typing methods and molecular characteristics are further discussed in the "Clonal Complexes" section.

The infectious dose of *L. monocytogenes* can vary with the strain, food type and the susceptibility of the infected individual. Virulence factors include two major internalins (A and B), pore forming toxin listeriolysin O, two phospholipases PlcA and PlcB and actin polymerisation factor ActA (Cossart *et al*., 2011). Pathogenicity involves the bacteria invading the gastrointestinal epithelium. Once inside the cell, Listeria uses listeriolysin O to escape from the host vacuole and reach the cell cytosol. Actin based motility allows the bacteria to move intracellularly and from cell to cell. From there the bacteria can enter the host's blood system and proliferate. Most virulence factors are regulated by PrfA, a transcriptional regulator (A de las Heras *et al*., 2011). Internalin A and B are involved in entry of the bacteria into cells via cadherins transmembrane proteins and Met receptors. Internalin A is a key virulence factor and loss of internalin A function may alter the phenotype (Jacquet *et al*., 2004, Nightingale *et al*., 2005, Van Stelten *et al*., 2016). Approximately 50% of *L. monocytogenes* food isolates have a mutation in the *inl*A gene (Figure 1). Epidemiology data has indicated that not all *L. monocytogenes* strains are equally virulent, differences in virulence among strains may exist (Hain *et al*., 2007). Thirty premature stop codons have been identified to date by Institut Pasteur (inlA_PMSC.xlsx (pasteur.fr)). The majority of *inlA* premature stop codons generate truncated secreted proteins which results in attenuated mammalian virulence. In addition to truncated proteins there may be a complete loss of *inlA* activity due to a

nucleotide deletion in a 5' homopolymeric tract (specific to Lineage II) that creates a frameshift mutation leading to a premature stop codon (Lomonaco *et al*., 2015). This potentially could affect virulence, making the bacteria avirulent for some individuals.



Jacquet *et al*., 2004

Figure 1. Truncated/full length Internalin A structure. Examples of full length and truncated forms for internalin expressed by *Listeria monocytogenes* (InlA1 to InlA4). InlA1 demonstrates full-length internalin, comparable to the reference strain EGD. InlA2 is a carboxy-terminal truncated internalin, similar to that produced by the LO28 strain. InlA3 is a truncated internalin, shorter than the LO28 strain. InlA4 is another example of a truncated version, larger than InlA2 or InlA3 but shorter than InlA1, the full-length internalin. InlA1 is the only example that promotes entry of *L. monocytogenes* into host cells expressing human E-cadherin. The truncated forms of InlA2, InlA3 and InlA4 cannot facilitate entry into host cells.

Bile

Bile is a yellow/green fluid produced by the liver in most vertebrates. In humans, bile is stored in the gall bladder where it is concentrated 5 to 10-fold through the removal of water and electrolytes (Dowd *et al*., 2011). Bile is composed mainly of water and constituents including bile acids, cholesterol, phospholipids and pigments bilirubin and biliverdin (Begley *et al*., 200b). The primary function of bile is to act as a biological detergent for the emulsification and solubilisation of fats. Bile also has antimicrobial properties that can affect the phospholipids and proteins of bacterial cell membranes and disrupt cellular homeostasis (Begley *et al*., 2005b). On digestion of food the gallbladder secretes bile through the production of a digestive hormone, cholecystokinin, into the duodenum of the small intestine.

Bile acids constitute a large proportion of bile organic components at approximately 50% (Begley *et al*., 2005b). They are synthesised in liver hepatocytes from cholesterol through a multienzyme process. These bile acids are termed primary bile acids, namely cholic and chenodeoxycholic acid. Approximately 300 to 600 mg of bile acids are produced daily to replace bile acids excreted in the faeces. Before secretion from the liver the bile acids are conjugated with one of two amino acids, glycine (glycoconjugated) or taurine (tauroconjugated). The ratio of glycoconjugates to tauroconjugates in human bile is approximately 3 to 1 (Begley *et al*., 2005b). These conjugated bile acids are often referred to as bile salts as they are completely ionised at physiological pH whereas unconjugated bile acids are not. The average concentration of glycoconjugate bile acid (GDCA) and tauroconjugated bile acid (TDCA) in the human GI tract is estimated at 5mM (Begley *et al*., 2005b, De Boever *et al*., 1999).

Secondary bile acids, deoxycholic acid and lithocholic acid, are produced from primary bile acids in the large intestine (caecum and colon) through the action of bacteria. The three main alterations include deconjugation, 7α-dehydroxlation and 7α-dehydrogenation (Begley *et al*., 2005b). Cholic acid is converted into deoxycholic acid and chenodeoxycholic acid into lithocholic acid. All four bile acids can be reabsorbed into the blood stream, returned to the liver and re-secreted in a process

called enterohepatic circulation (Russell., 2003). Bile acids are amphipathic with hydrophobic/hydrophilic regions and form micelles with phosphatidylcholine and cholesterol to aid digestion by emulsifying and solubilising fats. Bile acid also function to eliminate cholesterol from the body, emulsify fat soluble vitamins to enable absorption and have antimicrobial properties (Hofmann., 1999).

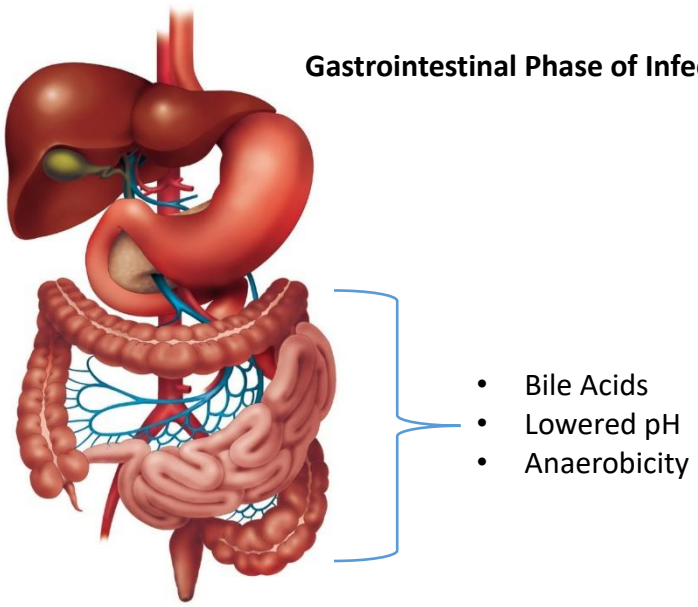


Figure 2. The human gastrointestinal tract. *L. monocytogenes* are exposed to various stressors in the GI tract including a lowered pH, anaerobic conditions and bile acids.

L. monocytogenes must survive a number of stressors to infect/colonise the human GI tract including acidic conditions in the stomach, bile in the GI tract and low oxygen availability/anaerobic conditions (White *et al*., 2015). *L. monocytogenes* is exposed to bile at three key stages during infection in vivo; in the GI tract during initial infection, in the liver where it can replicate and, in the gallbladder, where it can return to the intestine and subsequently to the environment (Quillin *et al*., 2011). The gastrointestinal phase of Listeria infection (Figure 2) is not as well studied as other bacteria and information on bile resistance is limited. The antimicrobial action of bile includes cell membrane damaging effects and disturbing macromolecule stability. Bile acids can induce DNA damage and secondary structure formation in RNA (Begley *et al*., 2005b). Gram-positive bacteria have been reported to be more sensitive to bile than Gram-negative bacteria. Bile resistance has also been reported to be strain specific and cannot be generalised by species (Begley *et al*., 2005a, Olier *et al*., 2004).

Begley *et al*., 2002 reported that *L. monocytogenes* strain LO28 is capable of tolerating concentrations of bovine, porcine and human bile above the concentration of bile encountered in vivo. Porcine bile, which contains trihydroxyconjugated bile salts is more potent than bovine bile which contains dihydroxyconjugated bile salts. Porcine bile is more akin to human bile in terms of bile salt/cholesterol, phospholipids/cholesterol and glycine to taurine ratios (Begley *et al*., 2005b). Analysis of human and porcine bile by High Pressure Liquid Chromatography (HPLC) demonstrates the conjugation of bile salts to glycine instead of taurine is more prevalent in both at 60 to 70% for humans and 85% in porcine (Legrand-Defretin *et al*., 1991, Rossi *et al*., 1999). The majority of studies investigating *L. monocytogenes* bile tolerance use reconstituted powdered bile extract. Dried bile extracts are prepared by extracting the soluble components of bovine or porcine bile with 80% alcohol and dried. Analysis of the dried extract using thin-layer chromatography specifies the presence of glycocholic acid at a level of 20 to 30%, taurocholic acid at 30 to 40% and taurodeoxycholic acid at 4 to 7% (Dowd *et al*., 2011). The process to produce the dried extract may affect the bile composition by causing precipitation of specific components such as inorganic electrolytes, proteins and binary lipids (Gruy-Kapral *et al*., 1999).

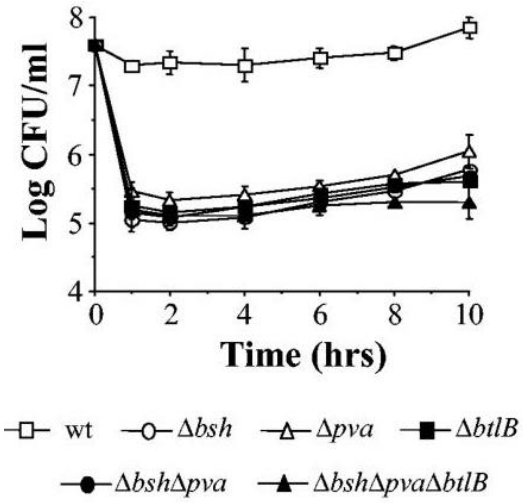
A study completed by Dowd *et al*., 2011 demonstrated that *L. monocytogenes* grew at the same rate as the non-pathogenic strain *Listeria innocua* in porcine gallbladder bile (pH 7). However, when the pH of gallbladder bile was reduced to pH 5.5 to simulate the release of bile in the small intestine, the toxicity of the gallbladder bile increased significantly and specific resistance mechanisms, including the general stress-response regulator Sigma factor B (*sigB*) (Begley *et al*., 2005a, Dowd *et al*., 2011), *bsh* (Begley *et al*., 2005a, Dussurget *et al*., 2002) and the bile exclusion system (*bilE*) (Sleator *et al*., 2005), were required for survival of the bacteria. In humans, bile mixes with chyme from the stomach as it is released and reduces the pH to as low as pH 5.2 (Campbell., 2009). This process identifies differences between bile in the gallbladder and bile within the small intestine, where antibacterial effects are increased (Dowd *et al*., 2011). Begley *et al*., 2005a demonstrated that deletion of the *bsh* gene results in a 2 log-unit reduction after exposing bacteria to 30% Oxgall

(bovine bile) and reduces the ability to colonise the GI tract in animal models. It was also shown that the deletion of a gene encoding a membrane located bile exclusion system (*bilE*) resulted in a 4-log reduction. Glycoconjugated bile acids demonstrate increased antibacterial effects at low pH and might be responsible for the inhibitory effects when the pH of gallbladder bile is decreased (Begley *et al*., 2005a, Dowd *et al*., 2011). Other genes shown to be involved in bile resistance include *btlB*, *sigB*, *pva*, *prfA* and *btlA*. Olier *et al*., 2004 reported that *L. monocytogenes* could grow in broth supplemented with 5% porcine bile salt. However, the toxicity of glycoconjugated bile salts was strongly pH dependent with toxicity increasing as the pH decreased. Contradictory results were reported by King *et al*., 2003 where the addition of 0.3% bile salts had a much greater effect on the exponential and stationary phase of growth. Differences may be explained by variation in experimental design.

The principal carnitine transporter OpuC has a role in the survival of *L. monocytogenes* in high salt concentrations and low temperature environments (Sleator *et al*., 2001). OpuC has also been suggested to play a significant role in *L. monocytogenes* bile tolerance in the small intestine, identified during studies investigating oral infection in mice (Watson *et al*., 2009). The multidrug efflux pumps MdrM and MdrT are induced by the bile salt, cholic acid. Studies have shown that MdrT can export cholic acid, and *mdrT* mutants are significantly attenuated both in vitro when exposed to cholic acid or bile, and in vivo in the gallbladders and livers of infected mice (Quillin *et al*., 2011). Other efflux pumps may have the potential to influence bile tolerance. Jiang *et al*., 2019, demonstrated that MdrL, is involved in the susceptibility of *L. monocytogenes* EGDe to benzalkonium chloride (BC), a disinfectant commonly used in food processing environments. The study demonstrated that growth of *mdrL* mutants was reduced when exposed to a sublethal concentration of BC and had a lower survival rate than the wildtype on exposure to a lethal concentration of BC.

Begley *et al*., 2005a demonstrated an initial decline in *L. monocytogenes* wild type strain (LO28) and *bsh*, *pva* and *btlB* mutants after a one-hour incubation in BHI broth supplemented with 30% oxgall. After this time, the bacteria adjusted to the

environment and after an extended lag phase, growth levelled off or increased, depending on the strain, over a 10-hour period (Figure 3).



Begley *et al*., 2005a

Figure 3. Role of *L. monocytogenes* LO28 *bsh*, *pva* and *btlB* genes in bile tolerance. Overnight cultures were inoculated into BHI broth supplemented with 30% oxgall. Cultures were enumerated using Ringer's solution and BHI agar at one-hour intervals over a ten-hour time period. Results observed an initial decline in numbers after one hour exposure to bile followed by an extended lag phase. After this, the bacteria began to acclimatise to the environment and numbers levelled off or increased, to varying degrees. Samples were tested in triplicate; error bars represent the standard deviations.

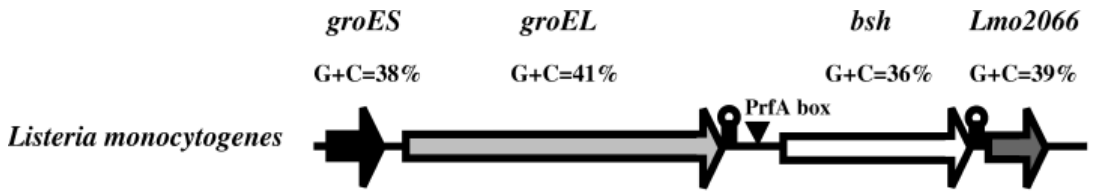
Bile Salt Hydrolase

Bile salt hydrolases (BSHs) are enzymes that deconjugate the primary bile acids, cholic acid and chenodeoxycholic acid. BSH is specific to commensal bacteria that inhabit the GI tract and a limited number of pathogens that infect via this route (Jones *et al*., 2008). They catalyse the hydrolysis of the amide bond between the C-24 position of the steroid moiety and the amino acid side chain of bile acids (Begley *et al*., 2005b). BSHs are oxygen insensitive and have an optimal pH between pH 5 and 6.

There are three proposed functions of microbial BSH (Begley *et al*., 2005b). The first theory is that deconjugation may liberate amino acids as nutritional sources of carbon, nitrogen and energy. BSH may also allow the incorporation of cholesterol or bile into bacteria cell membranes affecting host defence molecules. The function of BSH enzymes may include a role in bile tolerance by allowing bacteria to detoxify bile and aid survival in the gastrointestinal tract. BSH detoxifies bile by deconjugating the glycine/taurine side chain from the cholesterol core. The unconjugated bile acids precipitate out and BSH activity can be assessed using an agar plate assay based on this characteristic (Dashkevicz and Feighner., 1989).

L. monocytogenes can use bile salt hydrolase to detoxify bile. A study completed by Dussurget *et al*., 2002 demonstrated the presence of a *L. monocytogenes* specific gene encoding a bile salt hydrolase and deletion of the gene resulted in decreased resistance to bile for *L. monocytogenes*. The study also showed that BSH activity increased in a low oxygen environment, a condition that *L. monocytogenes* is exposed to in the GI tract and the gene is specific in pathogenic Listeria. The *bsh* gene encodes a functional intracellular enzyme in all pathogenic *Listeria* species and is positively regulated by PrfA, the transcriptional activator of known *L. monocytogenes* virulence genes (Dussurget *et al*., 2002). Analysis of the *L. monocytogenes* genome identified a 975 base pair open reading frame (ORF) and was 67% identical (219/324 amino acids) to *Lactobacillus plantarum* BSH (EC 3.5.1.24). The *bsh* gene (Figure 4) is preceded by the *groES–groEL* operon and followed by an ORF of unknown function, *Lmo 2066*, with both genes in the same orientation (Dussurget *et al*., 2002).

Genetic analysis of sixty isolates from countries including China, Canada, Switzerland, USA and Italy identified the presence of *bsh* genes in all *L. monocytogenes* isolates (Shi *et al*., 2021).



Dussurget *et al*., 2002

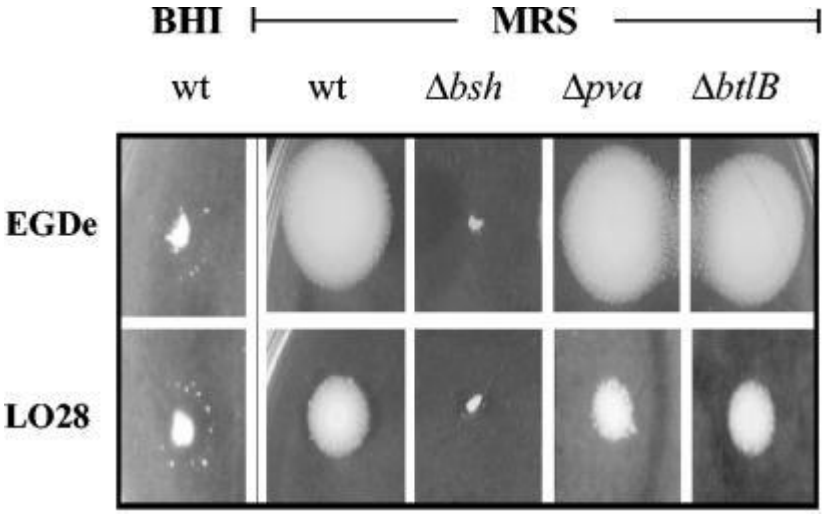
Figure 4. *L. monocytogenes bsh* gene region. *L. monocytogenes* complete genome sequence was used to prepare the figure to scale (approximately). Arrows indicate the orientation of the genes. Hairpins depict putative terminators. The black triangle identifies the PrfA box. The G+C % is specified by each gene name. The open reading frame *Lmo 2066* is an orthologue.

The alternative Sigma factor, Sigma B, is the predominant regulator of virulence genes (including *inlA, inlB*, *inlH*, and *bsh*) and survival factors in the GI tract, with PrfA acting as the main regulator of virulence genes in blood (Toledo-Arana *et al*., 2009, Gahan *et al*., 2014). Begley *et al*., 2005a and Zhang *et al*., 2011, investigated the role of *sigB* and *prfA* in *L. monocytogenes* bile tolerance. Zhang *et al*., 2011 demonstrated that *sigB* deletion significantly reduced the minimum inhibitory concentration (MIC) of bile salts for EGD *sigB* deletion mutants and EGD *pfrA* and *sigB* deletion mutants (2.6% and 2.2% vs 3.5% in wild type strain EGD). Growth rates were most affected by 3% bile salt. The *sigB* gene had more influence on *bsh* gene expression than *prfA*. Isolates with mutations in the SigB operon, which encodes the stress-inducible Sigma factor, Sigma B, were found to have reduced SigB activity, were more tolerant to salt stress and had less tolerance to low pH stress (Wu *et al*., 2022). SigB is vital for *L. monocytogenes* to establish infection in the host by facilitating stress responses to acid and bile in addition to regulating the *inlAB* operon to allow attachment to the host intestinal epithelial cells (Wu *et al*., 2022, Garner *et al*., 2006).

An agar plate assay was developed by Dashkevicz and Feighner., 1989 to detect bile salt hydrolase activity in Lactobacillus cultures. BSH activity caused hydrolysis of conjugated bile acids to the unconjugated form and presented as zones of precipitation around colonies or the formation of opaque granular white colonies on the agar plates. Lactobacillus selective MRS agar or Rogosa SL medium supplemented with taurodeoxycholic, taurocholic, or taurochenodeoxycholic acids (0.5% wt/vol) was used in the assay. The assay was dependent on the presence of bile salt hydrolase activity and the ability of the bacteria to acidify the agar to protonate free bile acids (Dashkevicz and Feighner., 1989). Results from the agar plate assay for BSH activity were verified by thin-layer chromatography.

Begley *et al*., 2005a utilised the BSH agar plate assay for *L. monocytogenes* cultures to assess BSH activity. Larger zone sizes indicated increased BSH activity. *L. monocytogenes* LO28 and EGDe were inoculated onto BHI agar supplemented with glycoconjugated bile acid (GDCA). Both strains resulted in a positive result for BSH activity presenting as translucent halos with small irregular precipitates around the patch inoculum. Repeating the assay using MRS agar resulted in increased zones of BSH activity around the inoculums. However, the zone of precipitation around the LO28 strain was much smaller than that of EGDe and did not correlate with the bile tolerance profiles, which indicated that LO28 had a higher bile tolerance in comparison to EGDe. Olier *et al*., 2004 also reported that LO28 was more bile tolerant than EGDe. A selection of other *L. monocytogenes* strains were also tested for BSH activity and the results did not correlate with their bile tolerance profiles. *bsh* gene mutants were not able to hydrolyse agar plates supplemented with GDCA or TDCA and signifies that the *bsh* gene encodes an enzyme that can deconjugate both glycoconjugated and tauroconjugated bile salts (Figure 5). The study also showed that *pva* and *btlB* gene deletion mutants did not affect hydrolysis of either bile salt GDCA or TDCA (Begley *et al*., 2005a). The roles of *prfA* and *sigB* were also assessed by Begley *et al*., 2005a using the agar plate assay. The LO28 and EGDe *prfA* deletion mutant zones were smaller than those of the wild-type on agar supplemented with GDCA/TDCA. The *sigB* gene mutants recorded no BSH activity indicating that SigB plays a greater role in *bsh* regulation than PrfA. Dusserget *et al*., 2002

demonstrated similar results using the BSH agar plate assay. An EGDe *bsh* deletion mutant showed no BSH activity when plated on 0.5 % (wt/vol) GDCA MRS agar. The wild-type EGDe resulted in a positive zone of BSH activity.



Begley *et al*., 2005a

Figure 5. BSH activity of *L. monocytogenes* LO28 and EGDe wildtype and *bsh*, *pva*, *btlB* mutants. Cultures were patch inoculated onto BHI or MRS agar supplemented with 0.5% (wt/vol) GDCA and incubated anaerobically for 48 hours. BSH activity presented as white halos of unconjugated bile acids around the cultures. BSH activity was not recorded for the *bsh* mutants.

Khodakivskyi *et al*., 2021 describes a novel enzymatic based probe assay with bioluminescent imaging to quantify BSH activity. Levels of bioluminescent light equate to BSH activity and is termed "BSH-activatable luciferin" (BAL). Sample types suitable for this method include pure enzymes and bacteria, intact faecal slurries and non-invasive imaging in live animal. It can also assess BSH activity in the gastrointestinal tract of mice and humans. A series of BSH-sensitive probes were developed to release a bioluminescent or fluorescent reporter if bacterial BSH is present. A specific bile acid is conjugated to a D-aminoluciferin scaffold (luciferin) via an amide bond that is specifically cleaved by the BSH enzyme. Cleavage of this bond results in uncaging of free luciferin, which can react with luciferase for light production which equates to BSH activity. Four BAL probes were developed for use

in the assay, cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and lithocholic acid (LCA). To investigate BSH selectivity in live bacterial cultures, the four probes were tested with a wild-type and a *bsh* deletion mutant of *Lactobacillus plantarum* WCFS1. Results indicated that the deletion of the *bsh* gene caused a complete inhibition of BSH activity (Khodakivskyi *et al*., 2021). Brandvold *et al*., 2018 described a similar approach to quantify bile salt hydrolase activity using a fluorescence assay. The study used a continuous fluorescence assay to characterise BSH activity with purified protein, cell lysates, whole cells and human GI samples. The method is based on a turn-on reporter strategy using a synthetic substrate that yields a fluorescent product depending on BSH activity. A chemical probe was designed that is a conjugate of cholic acid and an aminocoumarin fluorophore. Results demonstrated when the probe was added to a buffer containing purified BSH, a fluorescence signal was observed. Control samples using an unrelated hydrolase resulted in no increase in fluorescence demonstrating the selectivity of the assay.

Anaerobic Stress

L. monocytogenes, a facultative anaerobe, is exposed to a lowered oxygen availability in numerous environments including in soil, foods, decaying plant material and in the host gut. The ability of *L. monocytogenes* to establish infection in the host is dependent on overcoming stressors encountered in the GI tract including bile and a lowered oxygen availability. The effect of anaerobic conditions on the growth of *L. monocytogenes* is not as well understood or characterised as aerobic growth. Studies suggest a link between anaerobiosis and virulence, indicating that anaerobic conditions could be an environmental signal that induces virulence gene expression (Müller-Herbst *et al*., 2014).

Müller-Herbst *et al*., 2014 describes the oxygen dependent expression of the *L. monocytogenes* respiratory sequence. *L. monocytogenes* generates ATP via oxidative phosphorylation or substrate level phosphorylation. A requirement for ATP synthesis by oxidative phosphorylation via F_1F_0-ATP synthetase, a multi-subunit enzyme system, is the establishment of a proton motive force via the respiratory sequence. Electrons from NADH dehydrogenases, glycerol 3-phosphate dehydrogenase and, possibly, a putative formate dehydrogenase are transferred via menaquinone to either the cytochrome aa_3 or the cytochrome *bd* terminal (mena)quinol-oxidases. Transcription of several of the genes is regulated depending on oxygen availability. The expression of the respiratory sequence is downregulated without oxygen as the terminal electron acceptor, signifying a lower ATP yield and as a result, decreased growth of *L. monocytogenes*. It has been demonstrated that the anaerobic growth rate of *L. monocytogenes* EGDe, a commonly used laboratory reference strain, decreases when compared with aerobic growth (Müller-Herbst *et al*., 2014).

The alternative Sigma factor, SigB, which is involved with regulation of the *bsh* gene, demonstrates increased expression under anaerobic conditions (Begley *et al*., 2002). Anaerobiosis may activate the SigB regulon within the intestine and play a role in preparing the bacteria for tolerance to stressors in the gut. Additionally, expression of *sigB* is upregulated during infection of the murine gastrointestinal tract and may be influenced by anaerobic growth conditions.

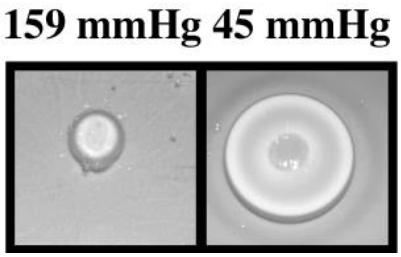
Proteomic analysis of *L. monocytogenes* reaction to bile salts under anaerobic conditions identified modifications to cell wall associated proteins, DNA repair proteins, protein folding chaperones and oxidative stress-response proteins (Payne *et al*., 2013). Increases in cell membrane branch-chain fatty acids were reported when *L. monocytogenes* was cultivated under anaerobic conditions and growth under increased carbon dioxide levels (Jydegaard-Axelsen *et al*., 2004). An alcohol acetaldehyde dehydrogenase homolog in *Listeria monocytogenes,* Listeria adhesion protein (LAP), promotes bacterial adhesion to intestinal epithelial cells *in vitro*. Growth in anaerobic conditions promotes increased levels of *lap* transcript and protein secretion and enables increased LAP-mediated adhesion of *L. monocytogenes* to cultured intestinal cells (Burkholder *et al*., 2009).

A study was completed by Wright *et al*., 2016 to determine if reduced oxygen availability increased *L. monocytogenes* bile tolerance. Four strains representing three serovars were tested for bile tolerance using porcine bile extract. Three of the strains had increased viability after exposing the isolates to 10% porcine bile under anaerobic conditions. One strain had no difference in viability when tested under aerobic and anaerobic conditions indicating that the response may be strain specific. Two of the strains tested anaerobically had increased expression of proteins associated with cell envelope and membrane bioenergetics when assessed by proteomic analysis (Wright *et al*., 2016). Anaerobic growth conditions increased bile resistance in a study completed by White *et al*., 2015 but a significant increase in resistance was only observed in virulent strains at pH 5.5. The effect of bile under anaerobic conditions was linked to pH. The toxicity of bile increased at lower pH values. The effect of anaerobic conditions on bile resistance was not consistent and may indicate strain specificity. A study by Bo Andersen *et al*., 2007 observed a significantly higher prevalence of *L. monocytogenes*, with increased virulence, in the organs of infected guinea pigs when cultures were grown under oxygen reduced conditions prior to dosage. Higher numbers of the bacteria were also isolated from faecal samples after dosage with the bacteria cultivated under anaerobic conditions.

It has been demonstrated that, bile salt hydrolase, an enzyme that *L. monocytogenes* uses to detoxify bile, increases under anaerobic conditions (Wright *et al*., 2016,

Begley *et al*., 2005a). Experiments assessing the impact of anaerobic stress on bile salt hydrolase using the bile salt GDCA demonstrated that higher amounts of GDCA was deconjugated on plates incubated under ≈ 45 mmHg oxygen tension than in aerobically incubated plates at atmospheric oxygen tension (159 mmHg) as displayed in Figure 6. BSH activity increased under anaerobic conditions (Dussurget *et al*., 2002). Anaerobic conditions in the host gut may trigger bile resistance mechanisms to allow *L. monocytogenes* survive and proliferate.

The effects of anaerobic stress indicate that oxygen availability does influence bile resistance but the response may be strain-specific and stress response mechanisms may differ between strains.



Dussurget *et al*., 2002

Figure 6. Effect of oxygen tension on *L. monocytogenes* BSH activity. *L. monocytogenes* EGDe was patch inoculated onto MRS agar supplemented with 0.5% GDCA (wt/vol). Plates were exposed to 159 mmHg (atmospheric oxygen tension) or 45 mmHg oxygen tension at 37 °C. Increased BSH activity was observed under a lower oxygen tension (45 mmHg).

Clonal Complexes

L. monocytogenes is divided into four phylogenetic lineages of which lineage I and II contain multiple clonal groups of public health significance (Nightingale *et al*., 2005, Orsi *et al*., 2011). Lineage I strains are more commonly associated with human listeriosis outbreaks. Lineage I is less prone to recombination and may have mechanisms that limit the acquirement of foreign DNA through horizontal gene transfer (Orsi *et al*., 2011). Lineage II has a higher genetic diversity which may be a factor in the adaption of Lineage II strains to diverse environments (den Bakker *et al*., 2008, Orsi *et al*., 2011). Lineage II isolates have been observed to carry more plasmids, often providing resistance to toxic metals and other environmental compounds. Lineage II isolates have also been reported to have mutations in *inlA* and *pfrA*. *InlA* is required for attachment to human host cells, mutations can lead to premature stop codons. This results in attenuated virulence for the strain. Strains with reduced pathogenicity, as a result of truncated and non-functional virulence factors (*inlA*), are commonly found in food isolates but are rarely found in human clinical cases (Nightingale *et al*., 2008, Orsi *et al*., 2011, Maury *et al*., 2016). Listeriolysin S haemolysin, a peptide haemolysin, is found in a section of lineage I isolates and is not present in lineages II, III, or IV isolates. Differences between the lineages may account for factors including survival in food/food processing environments and establishment of infection in the host.

Molecular typing methodologies are important in *L. monocytogenes* surveillance and outbreak/public health investigations. Traditionally, methods including Pulsed Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing (MLST) and Multi Virulence-Locus Sequence Typing (MVLST) are used to characterise *L. monocytogenes* strains. PFGE, a DNA based typing method was considered the gold standard to trace outbreaks associated with *L. monocytogenes*. MLST characterises sequence variation in seven housekeeping genes, namely *acbz* (ABC transporter), *bglA* (beta-glucosidase), *cat* (catalase), *dapE* (Succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (lactate dehydrogenase), *lhkA* (histidine kinase) (Ragon *et al*., 2008). MLST has moderate to high discriminatory power that may be too low for outbreak investigations (Moura *et al*., 2016). MVLST

targets six virulence genes, *prfA* (listeriolysin positive regulatory protein), *inlB* (internalin B), *dal* (alanine racemase), *lisR* (two-component response regulator), *inlC* (internalin C), *clpP* (clp protease proteolytic subunit), as described by Zhang *et al.,* 2004. Additional virulence genes have also been included in MVLST analysis, *inlA* (internalin A) and *actA* (actin A) (Chen *et al*., 2007, Ragon *et al*., 2008). MVLST can be employed in epidemiological typing (Lomonaco *et al*., 2015). The use of next generation sequencing technologies such as Whole Genome Sequencing (WGS) has become the new gold standard approach for epidemiology and population genetic study of *L. monocytogenes* (Lakicevic *et al*., 2023). The method can be used to identify genetic differences between strains to a higher level than other methods.

MLST further divides *L. monocytogenes* strains into clonal complexes. Clonal complexes (CCs) can be described as sequence types that are grouped by their similarity to a central allelic profile (genotype). They are groups of allelic profiles sharing six out of seven genes with at least one other member of the group (Maury *et al*., 2016). More than 170 clonal complexes have been identified (Lakicevic *et al.,* 2022). Several of these clonal complexes have been associated with epidemic clones responsible for multiple cases of listeriosis (Cantinellie *et al*., 2013). These CCs may be more virulent, have an increased presence in food processing environments and/or be more tolerant to stressors in food, food processing environments and the host. A study by Hingston *et al*., 2017 determined that *L. monocytogenes* tolerance to food-related stressors including salt, acid, cold and desiccation varies according to serotype and CC. 166 *L. monocytogenes* strains were phenotypically screened and results were correlated with genomic information. CC was a better predictor than serotype of salt and acid tolerance but not, cold and desiccation tolerance. Full length *inlA* was also observed to correlate with cold tolerance.

L. monocytogenes is a highly heterogenous species in relation to pathogenicity and comprises of hypervirulent and hypovirulent clones. Serious disease involving the central nervous system or materno-neonatal infection is typically associated with hypervirulent clones. Three categories of highly prevalent clones have been distinguished (Maury *et al*., 2016): infection associated clones isolated from clinical cases, CC1, CC2, CC4, CC6; food associated clones, CC9 and CC121 and intermediate

isolates including CC3, CC5, CC8 and CC16, CC37 and CC155 (Lakicevic *et al*., 2023). Several of the food associated CCs (e.g., CC9, CC121) are rarely associated with clinical listeriosis cases. Other CCs are commonly associated with clinical cases and are seldom isolated in food processing environments (e.g., CC1, CC4). Food related CC9 and CC121 are mainly isolated from highly immunocompromised patients. In comparison, CC1, CC2, CC4 and CC6 are predominantly isolated from patients without any immunity issues (Maury *et al*., 2016). CC121 is reported as the most commonly isolated CC from food samples (Knudsen *et al*., 2017).

Twenty-five of the clinical isolates used in this MSc study have previously been characterised using WGS (O'Callaghan *et al*., 2017, Hilliard *et al*., 2018). The strains were either Lineage I (14 isolates), serotype 4b, or lineage II (11 isolates), serotype 1/2a or 1/2c, comprising of 12 different clonal complexes. CC1 (7) and CC101 (4) were most prevalent and are primarily associated with clinical cases (Maury *et al*., 2016). CC121 accounted for three of the clinical strains. CC121 is mainly identified from food associated cases. The clinical isolates include two sets of mother and infant pairs. Analysis by single nucleotide polymorphism (SNP) revealed that they were highly related, with both pairs differing by 3 SNPs each. Two CC101 isolates, from separate patients, differed by just two SNPs indicating a phylogenetic link. They were isolated by the same local health authority within a one-month time period but no epidemiological link could be identified. The Irish clinical strains were also compared to a bank of strains isolated from Irish food and food processing environments (1300 isolates) using PFGE (Hilliard et al. 2018). 64% of the clinical PFGE profiles were previously found in food or in food processing environments highlighting the potential mode of transmission.

The aim of this study was to investigate the impact of bile and anaerobic stress on *L. monocytogenes* isolates. Bile is one of the stressors that *L. monocytogenes* encounters in the human gastrointestinal tract and must overcome to establish infection. Bile salt hydrolase (BSH) is an enzyme that *L. monocytogenes* can use to detoxify bile in order to establish infection. The study included isolates from food, environmental and clinical sources. A bile sensitivity assay and a BSH agar plate assay were developed to determine how sensitive the isolates were to bile and to

determine levels of BSH activity. Isolates were screened using conditions comparable to those in the human gastrointestinal tract, including a lowered pH and lowered oxygen availability. Results generated will provide insight into the effect of bile and growth environment on *L. monocytogenes* isolates and assess if there is a potential difference between the bile response of food/environmental and clinical isolates.

Materials and Methods

Bacterial strains, media and cultivation conditions

The bacterial strains used in this study are listed in Table 1. The bank of strains includes isolates from food, environmental and clinical sources. The lineage and clonal complex information for the isolates is displayed in Figure 7 with a further breakdown of food and environmental isolates in Figure 8 and clinical isolates in Figure 9. The *L. monocytogenes* isolates were stocked in BHI Broth with 20% glycerol and stored at -80 °C. To cultivate, Listeria stocks were streaked onto Brain Heart Infusion (BHI) Agar (Oxoid CM1135 37g/L supplemented with 1.5% agar Fisher Bioreagents Agar Powder BP-1423-500) and incubated at 37 °C for 18-24 hours. For liquid cultures an isolated colony from BHI agar was inoculated into 10 ml BHI broth and incubated statically at 37 °C for 18-24 hours. To generate aerobic conditions, broth cultures were grown in a shaking incubator for the time and temperature previously detailed. To generate anaerobic conditions, broth or plate cultures were incubated in an anaerobic gas jar with an anaerobic gas sachet (Merck Anaerocult A 113829) for the time and temperature previously detailed.

Table 1. List of *Listeria monocytogenes* strains (*Listeria species) and associated information, analysed in this study.

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
EGDe	-	Moorepark	Clinical	1/2a	II	ST-35	CC9
L. innocua*	-	VFSL	Reference Culture NCTC	N/A	N/A	N/A	N/A
944	19/03/2013	Moorepark	Food - Dairy – Cheese	4b	I	-	-
946	19/03/2013	Moorepark	Environment - Bin/Maturing Room	1/2a	II	ST-7	CC7
948	19/03/2013	Moorepark	Environment - Cutting Area Table	1/2a	II	ST-20	CC20
950	19/03/2013	Moorepark	Environment - Metal Frame	1/2a	II	ST-121	CC121
954	19/03/2013	Moorepark	Environment - Hall Corner	1/2c	II	ST-9	CC9
958	01/03/2013	Moorepark	Environment – Floor	1/2c	II	ST-9	CC9
960	01/03/2013	Moorepark	Food/Environment	1/2c	II	-	-
965	05/04/2013	Moorepark	Environment - Main Hall - Drain	1/2a	II	ST-9	CC9
966	01/03/2013	Moorepark	Environment – Sink	4b	I	ST-220	CC220
970	01/03/2013	Moorepark	Environment	-	I	ST-1	CC1
974	05/04/2013	Moorepark	Environment - Cutting Room – Wall	1/2a	II	ST-121	CC121
976	05/04/2013	Moorepark	Food - Mixed Foods - Stuffing (use by 13/11/13)	1/2a	II	ST-20	CC20
987	10/04/2013	Moorepark	Environment - Cutting Room – Shelf	1/2a	II	ST-121	CC121
991	11/04/2013	Moorepark	Food - Meat - Chicken Fillet (use by 2/4/13)	1/2a	II	ST-7	CC7
997	12/04/2013	Moorepark	Environment - Around Drain	1/2a	II	ST-37	CC37
1003	18/04/2013	Moorepark	Food - Meat - Chicken (use by 20/4/13)	1/2a	II	ST-7	CC7
1006	18/04/2013	Moorepark	Environment – Table	1/2a	II	ST-37	CC37
1013	20/05/2013	Moorepark	Environment - Drain in Warm Room	1/2a	II	ST-37	CC37

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
1019	20/05/2013	Moorepark	Food - Dairy - Raw Cheese 0420	1/2a	II	ST-37	CC37
1021	20/05/2013	Moorepark	Environment - Hall Corner – Broken	1/2c	II	ST-9	CC9
1023	01/05/2013	Moorepark	Food - Dairy - Unpasteurized Milk	4b	I	ST-220	CC220
1055	01/05/2013	Moorepark	Environment - Floor Press	1/2b	I	ST-59	CC59
1093	01/05/2013	Moorepark	Environment - Winch Room	1/2b	I	ST-59	CC59
1095	05/06/2013	Moorepark	Environment - Cutting Room – Mincer	1/2a	II	ST-37	CC37
1101	21/06/2013	Moorepark	Food/Environment	1/2a	II	-	-
1107	21/06/2013	Moorepark	Environment - Filleting Area- Floor around Drain	1/2a	II	ST-20	CC20
1109	21/06/2013	Moorepark	Environment - Filleting Area- Floor around Drain	1/2a	II	ST-20	CC20
1123	02/07/2013	Moorepark	Food - Seafood - Smoked Salmon Batch 141/13	1/2a	II	ST-121	CC121
1147	07/08/2013	Moorepark	Environment – Drain	1/2a	II	ST-7	CC7
1155	21/08/2013	Moorepark	Food - Meat - Sausages BB 13/8/13	1/2c	II	ST-9	CC9
1203	01/11/2013	Moorepark	Environment – Floor	1/2a	II	ST-18	CC18
1268	01/11/2013	Moorepark	Food - Dairy – Cheese	1/2b	I	ST-224	CC224
1304	-	NUIG	Environment	-	II	ST-836	CC155
1306	-	NUIG	Environment	-	II	ST-836	CC155
1309	-	NUIG	Environment	-	II	ST-204	CC204
1313	-	NUIG	Environment	-	II	ST-121	CC121
1317	-	NUIG	Environment	-	I	ST-2	CC2
1321	-	NUIG	Environment	-	II	ST-431	CC101
1370	-	EURL	Food – Meat	-	II	ST-9	CC9

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
1371	-	EURL	Food – Meat	-	II	ST-31	CC31
1372	-	EURL	Food – Meat	-	I	ST-3	CC3
1373	-	EURL	Food – Meat	-	II	ST-121	CC121
1374	-	EURL	Food – Meat	-	I	ST-3	CC3
1375	-	EURL	Food – Meat	-	I	ST-2	CC2
1376	-	EURL	Food – Meat	-	I	ST-3	CC3
1377	-	EURL	Food – Meat	-	I	ST-2	CC2
1378	-	EURL	Food – Dairy	-	I	ST-5	CC5
1379	-	EURL	Food – Dairy	-	II	ST-14	CC14
1380	-	EURL	Food – Meat	-	I	ST-2	CC2
1381	-	EURL	Food – Meat	-	I	ST-2	CC2
1382	-	EURL	Food – Dairy	-	I	ST-4	CC4
1383	-	EURL	Food – Dairy	-	II	ST-620	CC412
1384	-	EURL	Food-Seafood	-	I	ST-59	CC59
1385	-	EURL	Food-Seafood	-	II	ST-155	CC155
1386	-	EURL	Food-Seafood	-	I	ST-5	CC5
1387	-	EURL	Food-Seafood	-	I	ST-2	CC2
1388	-	EURL	Food-Seafood	-	I	ST-2	CC2
1389	-	EURL	Food-Seafood	-	II	ST-31	CC31
1390	-	EURL	Food – Dairy	-	I	ST-4	CC4
1391	-	EURL	Food – Dairy	-	I	ST-54	CC54
1392	-	EURL	Food – Seafood	-	I	ST-6	CC6
1393	-	EURL	Food – Meat	-	I	ST-2	CC2

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
1394	-	EURL	Food – Dairy	-	II	ST-26	CC26
1403	-	NUIG	Food – Seafood	-	II	ST-121	CC121
1411	01/03/2014	Moorepark	Environment – Bin	4b	I	ST-1	CC1
1413	01/03/2014	Moorepark	Environment – Floor	4b	I	ST-2	CC2
1423	01/03/2014	Moorepark	Environment	-	II	ST-121	CC121
1427	-	NUIG	Environment	-	II	ST-836	CC155
1428	-	NUIG	Environment	-	II	ST-836	CC155
1439	01/05/2014	Moorepark	Food - Seafood	-	II	ST-121	CC121
1441	01/05/2014	Moorepark	Environment - Pooled Water	-	I	ST-5	CC5
1445	01/05/2014	Moorepark	Food – Meat	-	II	ST-7	CC7
1502	-	NUIG	Environment	-	II	ST-836	CC155
1507	-	NUIG	Environment	-	II	ST-787	CC787
1513	01/05/2014	Moorepark	Food - Seafood – Salmon	1/2a	II	ST-121	CC121
1515	-	NUIG	Environment	-	II	ST-18	CC18
1519	-	NUIG	Food – Seafood	-	II	ST-121	CC121
1527	-	NUIG	Environment	-	II	ST-37	CC37
1534	01/07/2014	Moorepark	Food – Meat	-	I	ST-6	CC6
1564	01/07/2014	Moorepark	Environment – Drain	-	I	ST-2	CC2
1673	01/11/2014	Moorepark	Environment - Floor Farm	-	II	-	-
1679	01/11/2014	Moorepark	Environment – Box	-	II	ST-204	CC204
1734	01/01/2015	Moorepark	Environment – Floor	-	I	-	-
1798	01/03/2015	Moorepark	Food - Dairy – Milk	-	II	ST-18	CC18
1880	01/05/2015	Moorepark	Food - Vegetable – Mushroom	-	II	ST-8	CC8

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
1976	22/06/2015	Moorepark	Environment - Safemush	-	II	ST-8	CC8
1989	22/06/2015	Moorepark	Food - Seafood - 5048 3 Salmon	-	II	ST-121	CC121
1991	22/06/2015	Moorepark	Food - Seafood - 5062 b Salmon	-	II	ST-121	CC121
2075	01/07/2015	Moorepark	Environment - Safemush - 4.1.1 Drain – Entrance	-	II	ST-91	CC14
2095	02/07/2015	Moorepark	Environment - Safemush - 11.1.7 Drain - Outside Number 4	-	I	ST-54	CC54
2105	02/07/2015	Moorepark	Environment - Safemush - 11.1.5 Floor at Door of Fridge	-	I	ST-54	CC54
2113	02/07/2015	Moorepark	Environment – Safemush	-	I	ST-1	CC1
2181	25/08/2015	Moorepark	Environment - Safemush - 5.2.3 T 23 Gap	-	I	ST-220	CC220
2183	26/08/2015	Moorepark	Environment - Safemush - 8.2.9 Bay 8 Mixed 12/08/15	-	I	ST-2	CC2
2185	26/08/2015	Moorepark	Environment - Safemush - 4.2.7 Drain Water House 10	-	II	ST-91	CC14
2226	01/09/2015	Moorepark	Environment - Safemush - 13.2.2 1M Squared Floor Number 5	-	I	ST-220	CC220
2234	03/09/2015	Moorepark	Environment - Safemush - 1.2.5 Bin	-	I	ST-220	CC220
2237	03/09/2015	Moorepark	Environment - Safemush - 1.2.6 Ground at Door	-	I	ST-2	CC2
2240	03/09/2015	Moorepark	Environment - Safemush - 2.2.4 Floor Picking Trolley	-	I	ST-59	CC59
2241	03/09/2015	Moorepark	Environment - Safemush - 2.2.4 Floor Picking Trolley	-	I	ST-59	CC59

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
2256	03/09/2015	Moorepark	Environment – Safemush	-	II	ST-121	CC121
2259	03/09/2015	Moorepark	Environment – Safemush	-	I	ST-54	CC54
2294	01/09/2015	Moorepark	Environment - Safemush – Ladder	-	II	ST-8	CC8
2298	01/09/2015	Moorepark	Environment – Smoker	-	I	ST-808	CC379
2507	01/04/2015	Moorepark	Environment – Drain	-	I	ST-220	CC220
3442	-	NLRL	Food - Vegetable - 19-248 Kale	-	I	ST-87	CC87
3443	-	NLRL	Food - Vegetable - 18-0813 Salad Leaves	-	II	ST-18	CC18
3444	-	NLRL	Food - Vegetable - 18-0725 Radish	-	I	ST-1	CC1
3445	-	NLRL	Food - Vegetable - 18-1279 Spinach	-	I	ST-54	CC54
3446	-	NLRL	Food - Vegetable - 18-1280 Chard	-	I	ST-54	CC54
3447	-	NLRL	Food-Vegetable - 18-0723 Salad Leaves	-	I	ST-59	CC59
3448	-	NLRL	Food - Vegetable - 18-0812 Radish Shoots	-	II	ST-18	CC18
6179	-	Moorepark	Food – Dairy	-	II	ST-121	CC121
10403S	-	Moorepark	Clinical	-	II	ST-85	CC7
1513C	-	NUIG	Food - Environment	-	II	ST-787	CC787
F2365	11/10/2014	UCC	Food – Dairy	4b	I	ST-1	CC1
F1857-15	-	UCD	Environment	-	I	ST-5	CC5
F1994-15	-	UCD	Food – Meat	-	I	ST-220	CC220
F2442-15	-	UCD	Food/Environment	-	-	-	-
F2446-15	-	UCD	Food/Environment	-	-	-	-
F2299-15	-	UCD	Environment	-	I	ST-5	CC5
F347-16	-	UCD	Environment	-	I	ST-5	CC5
F365-16	-	UCD	Food – Meat	-	II	ST-121	CC121

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
F671-16	-	UCD	Environment	-	I	ST-5	CC5
F991-16	-	UCD	Environment	-	I	ST-5	CC5
F1209-16	-	UCD	Environment	-	II	ST-121	CC121
F1221-16	-	UCD	Food – Meat	-	II	ST-121	CC121
F1230-16	-	UCD	Food – Meat	-	II	ST-121	CC121
F1524-16	-	UCD	Environment	-	I	ST-5	CC5
F111-17	-	UCD	Food - Mixed Foods	-	II	ST-121	CC121
F112-17	-	UCD	Food - Mixed Foods	-	II	ST-121	CC121
F113-17	-	UCD	Food/Environment	-	-	-	-
F114-17	-	UCD	Food - Mixed Foods	-	II	ST-121	CC121
F168-17	-	UCD	Environment	-	II	ST-121	CC121
F340-17	-	UCD	Environment	-	II	ST-121	CC121
F345-17	-	UCD	Environment	-	II	ST-121	CC121
F347-17	-	UCD	Environment	-	II	ST-121	CC121
F348-17	-	UCD	Environment	-	II	ST-121	CC121
F742-17	-	UCD	Environment	-	I	ST-5	CC5
F1099-17	-	UCD	Food/Environment	-	-	-	-
F1109-17	-	UCD	Environment	-	II	ST-121	CC121
F1644-17	-	UCD	Environment	-	I	ST-5	CC5
F1646-17	-	UCD	Environment	-	II	ST-121	CC121
F2151-17	-	UCD	Food – Meat	-	II	ST-121	CC121
F2152-17	-	UCD	Food – Meat	-	II	ST-121	CC121
F2153-17	-	UCD	Food – Meat	-	II	ST-121	CC121

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
F2154-17	-	UCD	Food – Meat	-	II	ST-121	CC121
F2155-17	-	UCD	Food – Meat	-	II	ST-121	CC121
F2160-17	-	UCD	Food – Meat	-	II	ST-121	CC121
F2161-17	-	UCD	Environment	-	II	ST-121	CC121
F2165-17	-	UCD	Environment	-	II	ST-121	CC121
F2166-17	-	UCD	Environment	-	I	ST-5	CC5
F2170-17	-	UCD	Environment	-	II	ST-121	CC121
F2175-17	-	UCD	Food/Environment	-	-	-	-
F2176-17	-	UCD	Environment	-	I	ST-5	CC5
G-001	-	GUH	Clinical Isolate	-	-	-	-
G-002	-	GUH	Clinical Isolate	-	II	ST-121	CC121
G-003	-	GUH	Clinical Isolate	-	II	ST-8	CC8
G-004	-	GUH	Clinical Isolate	-	I	ST-1	CC1
G-005	-	GUH	Clinical Isolate	-	I	ST-2	CC2
G-006	-	GUH	Clinical Isolate	-	I	ST-5	CC5
G-007	-	GUH	Clinical Isolate	-	I	ST-1	CC-1
G-008	-	GUH	Clinical Isolate	-	I	ST-1	CC-1
G-009	-	GUH	Clinical Isolate	-	-	-	-
G-010	-	GUH	Clinical Isolate	-	II	ST-37	CC37
G-011	-	GUH	Clinical Isolate	-	I	ST-1	CC1
G-012	-	GUH	Clinical Isolate	-	I	ST-2	CC2
G-013	-	GUH	Clinical Isolate	-	I	ST-59	CC59
G-014	-	GUH	Clinical Isolate	-	II	ST-14	CC14

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
G-015	-	GUH	Clinical Isolate	-	I	ST-220	CC220
G-016	-	GUH	Clinical Isolate	-	II	ST-37	CC37
G-017	-	GUH	Clinical Isolate	-	II	ST-8	CC8
G-018	-	GUH	Clinical Isolate	-	I	ST-54	CC54
G-019	-	GUH	Clinical Isolate	-	-	-	-
G-020	-	GUH	Clinical Isolate	-	II	ST-37	CC37
G-021	-	GUH	Clinical Isolate	-	I	ST-1	CC1
G-022	-	GUH	Clinical Isolate	-	I	ST-6	CC6
G-023	-	GUH	Clinical Isolate	-	II	ST-226	CC226
G-024	-	GUH	Clinical Isolate	-	I	ST-3	CC3
G-025	-	GUH	Clinical Isolate	-	II	ST-8	CC8
G-026	-	GUH	Clinical Isolate	-	II	ST-504	CC475
G-027	-	GUH	Clinical Isolate	-	-	-	-
G-028	-	GUH	Clinical Isolate	-	II	ST-429	CC90
G-029	-	GUH	Clinical Isolate	-	-	-	-
G-030	-	GUH	Clinical Isolate	-	I	ST-2	CC2
G-031	-	GUH	Clinical Isolate	-	I	ST-6	CC6
G-033	-	GUH	Clinical Isolate	-	I	ST-2	CC2
G-034	-	GUH	Clinical Isolate	-	I	ST-54	CC54
G-035	-	GUH	Clinical Isolate	-	II	ST-121	CC121
G-036	-	GUH	Clinical Isolate	-	-	-	-
G-037	-	GUH	Clinical Isolate	-	I	ST-1	CC1
G-038	-	GUH	Clinical Isolate	-	II	ST-155	CC155

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
G-039	-	GUH	Clinical Isolate	-	II	ST-18	CC18
G-040	-	GUH	Clinical Isolate	-	I	ST-87	CC87
G-041	-	GUH	Clinical Isolate	-	I	ST-6	CC6
G-042	-	GUH	Clinical Isolate	-	I	ST-6	CC6
G-043	-	GUH	Clinical Isolate	-	I	ST-1	CC1
G-044	-	GUH	Clinical Isolate	-	-	-	-
G-045	-	GUH	Clinical Isolate	-	I	ST-2	CC2
G-046	-	GUH	Clinical Isolate	-	I	ST-1	CC1
G-047	-	GUH	Clinical Isolate	-	-	-	-
G-048	-	GUH	Clinical Isolate	-	II	ST-121	CC121
MQ130026	-	GUH	Clinical Isolate-Blood	4b	I	ST-1	CC1
MQ130029	-	GUH	Clinical Isolate-CSF	4b	I	ST-1	CC1
MQ130032	-	GUH	Clinical Isolate-Blood	4b	I	ST-1	CC1
MQ130033	-	GUH	Clinical Isolate-Blood	4b	I	ST-54	CC54
MQ130037	-	GUH	Clinical Isolate-Blood	1/2a	II	ST-18	CC18
MQ130042	-	GUH	Clinical Isolate-Pleural Swab	4b	I	ST-1	CC1
MQ130058	-	GUH	Clinical Isolate-Blood	4b	I	ST-6	CC6
MQ140011	-	GUH	Clinical Isolate-Placental Surface Swab-Mother/Infant	1/2a	II	ST-101	CC101
MQ140012	-	GUH	Clinical Isolate-Blood-Mother/Infant	1/2a	II	ST-101	CC101
MQ140025	-	GUH	Clinical Isolate-Ear Swab	4b	I	ST-1	CC1
MQ140029	-	GUH	Clinical Isolate-Blood	1/2a	II	ST-7	CC7
MQ140030	-	GUH	Clinical Isolate-CSF	4b	I	ST-4	CC4

Sample ID	Date	Source	Source Food/Environment/Clinical	Serotype	Lineage	Sequence Type	Clonal Complex
MQ140031	-	GUH	Clinical Isolate-Blood	4b	I	ST-1	CC1
MQ140032	-	GUH	Clinical Isolate-Blood	1/2a	II	ST-425	CC90
MQ140033	-	GUH	Clinical Isolate-Blood	4b	I	ST-1	CC1
MQ140034	-	GUH	Clinical Isolate-Blood-Mother/Infant	1/2a	II	ST-1349	CC121
MQ140035	-	GUH	Clinical Isolate-Ear Swab-Mother/Infant	1/2a	II	ST-1349	CC121
MQ150001	-	GUH	Clinical Isolate-Blood	1/2a	II	ST-37	CC37
MQ150004	-	GUH	Clinical Isolate-Placental Swab	4b	I	ST-54	CC54
MQ150005	-	GUH	Clinical Isolate-Blood	4b	I	ST-6	CC6
MQ150007	-	GUH	Clinical Isolate-CSF	1/2a	II	ST-431	CC101
MQ150008	-	GUH	Clinical Isolate-Blood	1/2a	II	ST-431	CC101
MQ150011	-	GUH	Clinical Isolate-Nasal Swab	1/2a	II	ST-20	CC20
MQ150012	-	GUH	Clinical Isolate-Blood	4b	I	ST-6	CC6
MQ150013	-	GUH	Clinical Isolate-Blood	4b	I	ST-2	CC2

VFSL: Veterinary Food Safety Laboratory, NUIG: University of Galway, EURL: European Union Reference Laboratory, NLRL: National Listeria Reference Laboratory, UCD: University College Dublin, GUH: Galway University Hospital

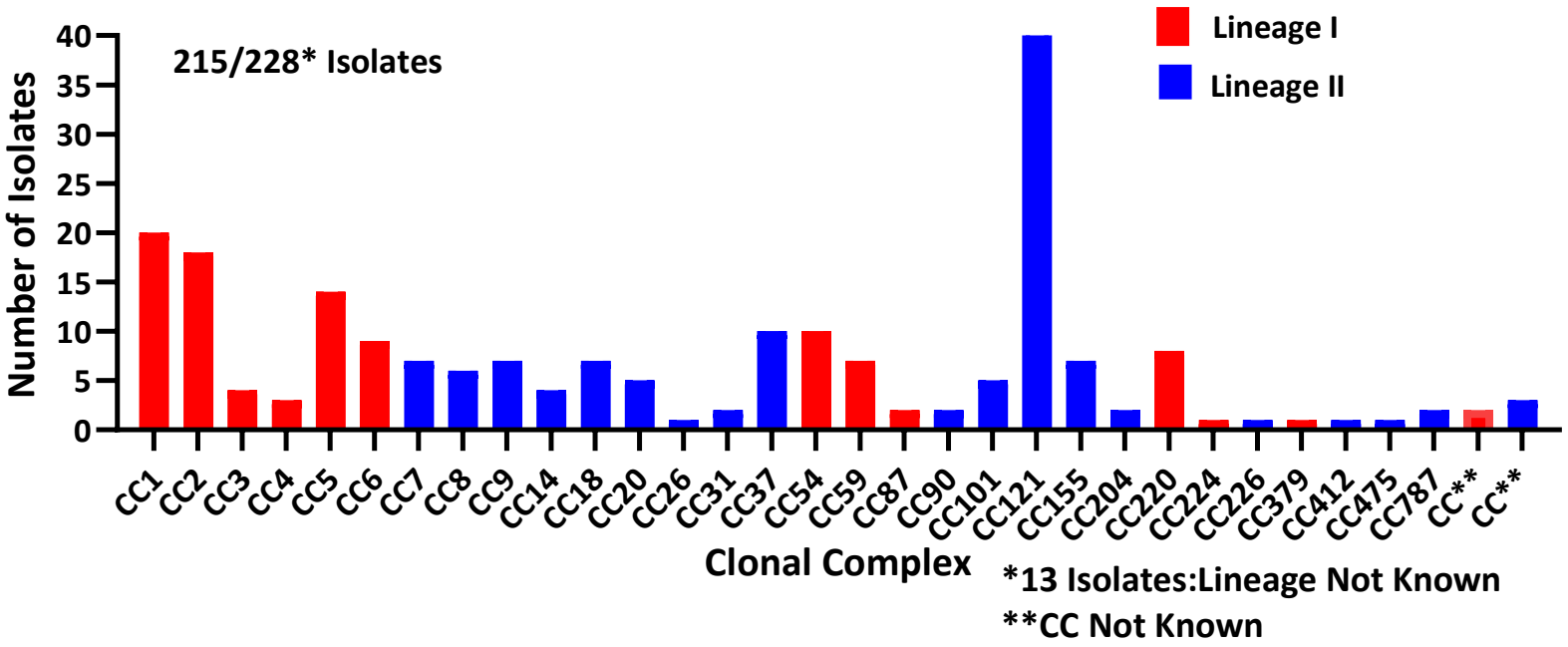


Figure 7. Number of **food, environmental and clinical isolates** by lineage and clonal complex group.

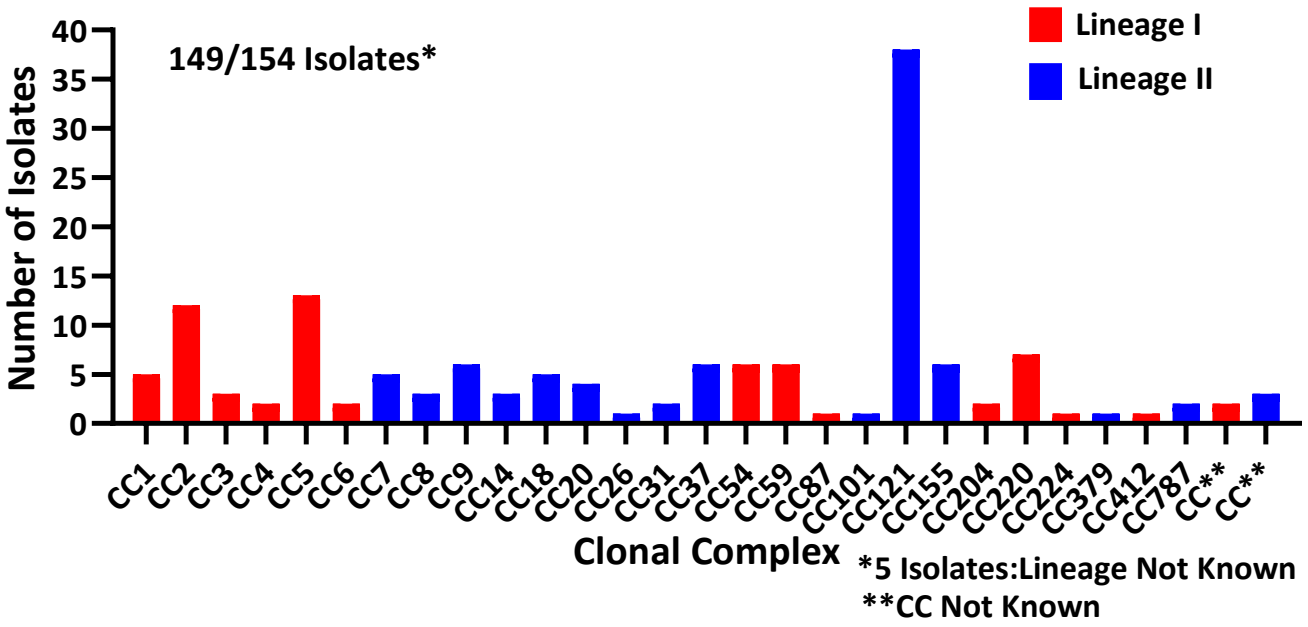


Figure 8. Number of **food and environmental isolates** by lineage and clonal complex group.

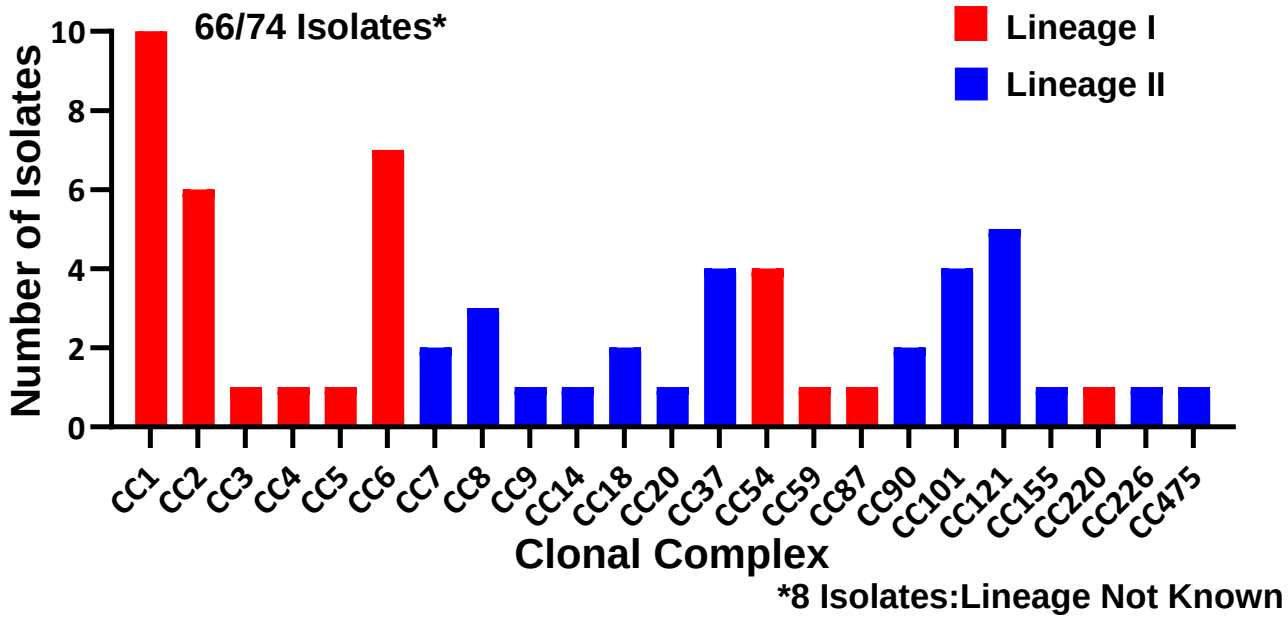


Figure 9. Number of **clinical isolates** by lineage and clonal complex group.

Bile Resistance Assay

Development Work

Selection of Bile

Different types of bile were trialled to assess the suitability to screen a large number of *L. monocytogenes* cultures (228 isolates). The bile types trialled:

- Dried purified ox-bile: Merck Millipore 70168 100g
- Purified bile acid TDCA (sodium taurodeoxycholate hydrate): Sigma T0875-1G)
- Dried porcine bile extract: Sigma B8631-100g

Development work included conditions to mimic those in the human gastrointestinal tract where bacteria are exposed to bile. Conditions in the GI tract include a lowered pH (approximately pH 5.5) and a lowered oxygen availability/anaerobic conditions. A 2% culture inoculum was trialled with the different bile broths. After bile exposure, samples were serially diluted using quarter strength Ringer's solution and enumerated using BHI agar.

Dried Purified Ox-Bile

To test the suitability of ox-bile, an 8% solution was prepared in pH 7.5/pH 5.5 BHI broth. An 8 to 10% solution of the dehydrated product equates to 1g of fresh bile (Information obtained from Ox-bile (sigmaaldrich.com)).

Purified Bile Acid TDCA

A 5mM concentration of TDCA was prepared using pH 7.5/pH 5.5 BHI broth. The bile acid was dissolved in sterile water and added to broth. 5mM equates to the average concentration of TDCA in the human GI tract.

Dried Porcine Bile Extract

Concentrations ranging from 1% to 10% were tested using pH 7.5/pH 5.5 BHI broth. The dried extract was added to BHI broth and mixed on a stirring plate until dissolved (higher concentrations did not solubilise well).

Bile Concentration

Different concentrations were assessed depending on the bile. A literature review established the equivalent human bile concentrations for the different bile types. All bile types trialled had solubility issues at higher concentrations (5% and 10%) and were difficult to maintain in solution. DMSO was added to assist with solubility but it was not suitable for the assay. To overcome this issue samples were periodically mixed to maintain solubility. The higher concentrations were included to determine the solubility potential of each bile type and were greater than the equivalent human bile concentrations.

Growth Medium/pH

BHI broth was used in the trial experiments to assess bile selection. Two different pH conditions were assessed: pH 7.5 and pH 5.5. To mimic conditions in the human gut, the pH of the broth was lowered from pH 7.5 to pH 5.5 using 1M hydrochloric acid (Sigma: formula weight of 36.46 g/mol and a concentration of 37 % w/w). The pH of the broth was adjusted to pH 5.5 before autoclaving at 121 °C for 15 minutes. The pH of the broth was checked after sterilisation to ensure the pH was maintained. The pH increased slightly after autoclaving, rising from pH 5.5 to pH 5.6/5.7. This was taken into account during the preparation of the broth to ensure the final pH was accurate (lowered the pH to 5.3/5.4 prior to autoclaving).

Growth Environment

Initial trials for development of the bile resistance assay were performed under standard atmospheric conditions i.e. static incubation at 37 °C. Once an appropriate bile was selected to screen the bank of Listeria strains, aerobic and anaerobic conditions were incorporated to assess the impact of growth environment.

Bile Exposure Time

Trials were performed using one- and two-hour bile exposure times at 37 °C. The time points were selected after reviewing results relating to bile tolerance from Begley *et al*., 2005a. (Figure 3).

Developed Bile Resistance Assay-Screen for *L. monocytogenes* Isolates

An isolated *L. monocytogenes* colony from a BHI plate (18 to 24-hour culture) was inoculated into 10 ml BHI broth pH 7.5 and incubated statically in an anaerobic jar with an anaerobic sachet at 37 °C for 18 to 24 hours for anaerobic cultures or shaking at 37 °C for 18 to 24 hours for cultures grown aerobically (Figure 10). After incubation, the Optical Density 600 nm was read for each sample to ensure comparable growth rates. Biological and technical triplicates were prepared for each sample. BHI agar plates were dried in a sterile cabinet for 15 minutes before use to enumerate Time 0 and Time 1 values. A 1% porcine bile extract BHI pH 5.5 broth was prepared by weighing 1 g of bile into 100 ml of broth. The solution was placed on a stirrer plate with a magnetic bar to solubilise the bile. Once solubilised, 5 ml of the bile broth was pipetted into 25 ml universal containers and inoculated with a 2% inoculum of the overnight anaerobic/aerobic culture. The bile broth was mixed and incubated statically at 37 °C for one hour. To enumerate the T0 sample, a 2% inoculum was inoculated into 5 ml pH 5.5 BHI broth (without bile). The sample was mixed and 100 µl was serially diluted in 900 µl quarter strength Ringer's solution to dilution 10^{-7}. 20 µl of dilutions T0 10^{-6} and 10^{-7} were spotted on BHI agar to enumerate. After one-hour exposure to bile, 100 µl of the bile broth was serially diluted in 900 µl Ringer's solution. 20 µl of T1 dilutions 10^{-3}, 10^{-4}, 10^{-5} were plated onto BHI agar. Plates were left to dry before inverting and incubating at 37 °C in standard environmental conditions for 18 to 24 hours. After incubation, colony counts between 3 and 30 were enumerated for both the T0 and T1 samples. An average value from the triplicate results was used to calculate the colony forming units/ml. The percentage bile resistance was calculated using the difference between T0 and T1 results. The higher the bile resistance percentage, the more bile tolerant the isolate.

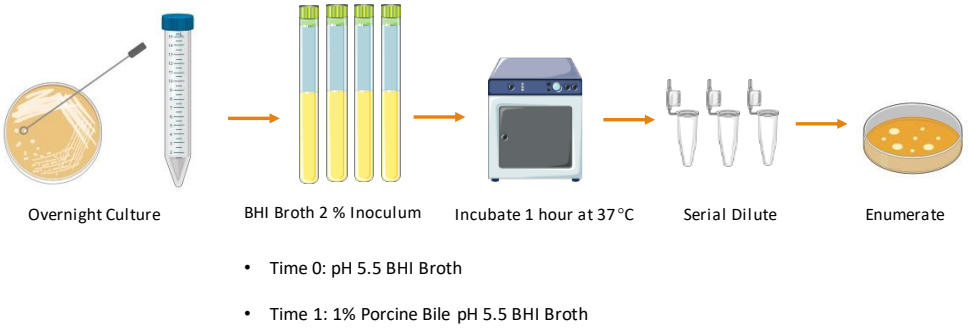


Figure 10. Bile Resistance Assay Schematic. Cultures were grown anaerobically or aerobically in BHI broth at 37 °C for 18 to 24 hours. Overnight cultures were inoculated into pH 5.5 BHI broth/1% bile pH 5.5 BHI broth for 1 hour at 37 °C. To enumerate, time 0 and time 1 samples were serially diluted using Ringer's solution and plated onto BHI agar.

Bile Salt Hydrolase Agar Plate Assay

Development Work

Selection of Agar

Testing of different parameters was required to develop the assay system to screen a large amount of *L. monocytogenes* cultures (228 isolates). BHI agar and de Man, Rogosa and Sharpe (MRS) agar were used to assess performance for the plate assay. The agars were supplemented with porcine bile (0.5% and 1% weight/volume) or purified bile acids, GDCA (Sigma G9910-5G) and TDCA (0.5% wt/vol). MRS agar was included as it is routinely used to detect BSH activity in Lactobacillus cultures. MRS agar does not support the growth of Listeria but it was included in the trial for Listeria BSH activity. Two different brands of MRS agar were tested, Oxoid MRS agar (CM0361) and Merck MRS agar (699-64). The Merck brand of MRS included the addition of Tween 80 before autoclaving, the Oxoid brand contained Tween in the powdered form. Bile agar plates were prepared and inoculated with 18-to-24-hour cultures. Different inoculation methods were also assessed using broth cultures and agar plate cultures. For the broth culture inoculum, 20 µl of an overnight culture was plated onto the bile agar. For the agar plate cultures, a sterile 100-200 µl micropipette tip was used to spot inoculate a small amount of culture onto the plate. Plates were incubated aerobically/anaerobically, depending on the condition being assessed and results were read after 48 and 72 hours.

Selection of Bile

Dried porcine bile extract and bile acids, GDCA and TDCA, were assessed for the assay. Bile/bile acids were dissolved in sterile water and added to cooled molten agar before pouring into petri dishes and allowing to solidify. For the porcine bile, concentrations of 0.5% and 1% (wt/vol) were tested. For the bile acids, a concentration of 0.5% (wt/vol) was used. The bile plates were inoculated with 18-to-24-hour cultures and incubated aerobically/anaerobically. Plates were assessed for BSH activity after 48 and 72 hours. BSH activity presented as white zones of

precipitation surrounding the culture. The zone size diameters were measured in millimetres (mm).

Environmental Conditions

Trials were carried out under aerobic and anaerobic conditions. For the aerobic plates, cultures were inoculated onto the bile agar and incubated at 37 °C in standard atmospheric conditions. For the anaerobic trial, bile plates were stored in anaerobic conditions (anaerobic jar and anaerobic sachet) for at least 72 hours before use. Inoculated plates were incubated at 37 °C in an anaerobic jar with an anaerobic sachet. Plates were read after 48 hours and 72 hours.

Developed Bile Salt Hydrolase Agar Plate Assay-Screening System for *L. monocytogenes* Isolates

To prepare the bile acid agar plates, 10 ml of cooled sterile MRS agar was poured into petri dishes and allowed to solidify. 0.5% (wt/vol) concentrations of GDCA/TDCA were prepared by dissolving in sterile water and added to cooled MRS agar before overlaying 10 ml over the first layer of MRS. Plates were allowed to dry before inverting and storing in an anaerobic jar with an anaerobic sachet for at least 72 hours before use. Listeria cultures were grown on BHI agar and incubated at 37 °C for 18 to 24 hours. Sterile 100-200 µl micropipette tips were used to patch inoculate a small amount of Listeria culture onto the bile acid plates: 0.5% TDCA MRS agar and 0.5% GDCA MRS agar. Eight different cultures were tested on the same plate. The patch inoculums were kept as uniform as possible to allow for a comparison of results between the different cultures. A BSH positive (*L. monocytogenes* EDGe) and negative culture (*L. innocua)* were tested with each batch of bile agar plates. Cultures were patched onto MRS agar without bile acids as controls. Samples were tested in duplicate. Plates were incubated at 37 °C in an anaerobic jar with an anaerobic sachet for 72 hours. Plates were read after 48 hours and 72 hours (Figure 11). Positive results for BSH activity presented as white zones of precipitation (deconjugated bile acids) surrounding the inoculum. Precipitation zone size diameters were measured in millimetres (mm) and recorded. The assay was developed as a semi-quantitative

assessment of BSH with zone size equated to levels of BSH activity. Larger zones indicated increased BSH activity, smaller zones indicated less BSH activity.

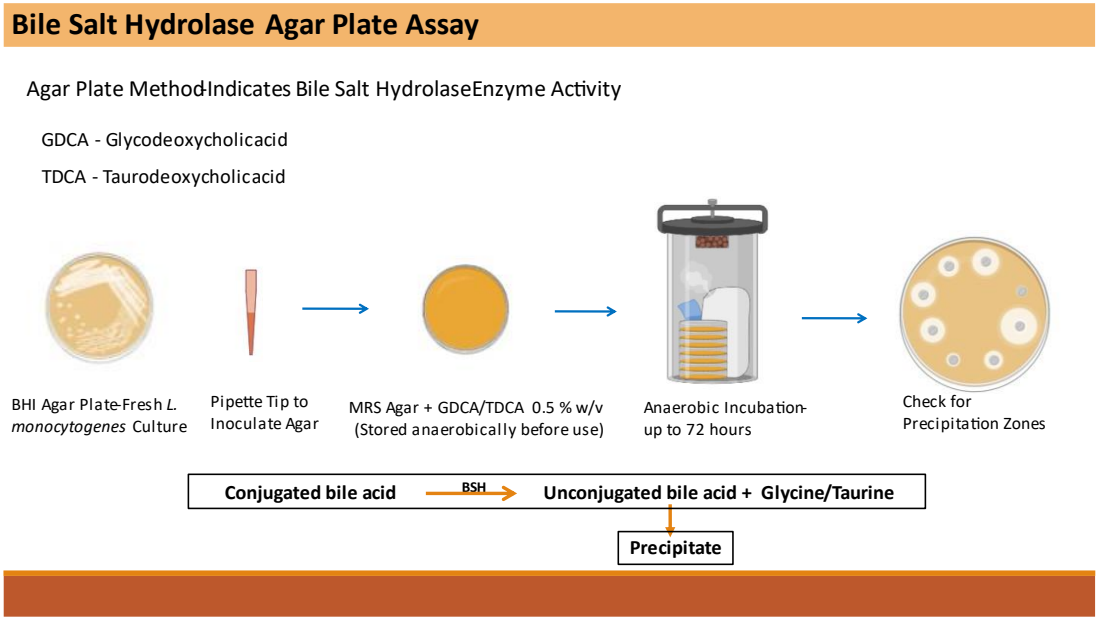


Figure 11. Bile Salt Hydrolase Agar Plate Assay Schematic. Cultures were patch inoculated onto MRS agar supplemented with 0.5% GDCA/TDCA (wt/vol). Plates were incubated anaerobically at 37 °C and read at 48 and 72 hours. White precipitate halos of deconjugated bile acids indicate BSH activity.

Bile Salt Hydrolase-Bioluminescence Probe Assay

Preparation of Cell Free Extracts

A colony of *L. monocytogenes* from a BHI agar plate was inoculated into 10 ml BHI broth and incubated at 37 °C for 18 to 24 hours. Overnight cultures were centrifuged at 5000 g at 4 °C for 15 minutes. The supernatant was discarded and the pellet was washed twice with 0.1 M phosphate buffer (pH 6.8-7.0). The suspension was transferred into a 1.5 ml microtube for further steps. Between each washing step, the suspension was centrifuged at 12,200 g for 60 seconds. The pellet was resuspended in 1 ml 0.2 M phosphate buffer and placed on ice. The microtube was placed in a beaker of ice and the suspension was sonicated with 7 cycles of 30 second 10 µm pulse followed by 30 seconds of cooling. After sonicating, the suspension was centrifuged at 12,300 g for 13 minutes at 4 °C. The supernatant was transferred to a new microtube and the cell free extract was used immediately or stored at -20 °C until required.

BSH Bioluminescence Probe Assay

The protocol was based on an assay developed by Professor Elena Goun and her team in the Institute of Chemical Sciences and Engineering EPFL, Switzerland. For use in the bioluminescence probe assay (Figure 12), the cell free extract protein concentrations were adjusted to between 1.0 and 1.25 mg/ml. 200 µl of the cell free extract was mixed with 200 µl of 40 µM enzymatic detection probe (cholic or deoxycholic) in mercaptoethanol. For control samples, cell free extract was mixed with 2 µM of aminoluciferin in mercaptoethanol. Samples were incubated at 37 °C for 1 hour before centrifuging at 13,000 g for 5 minutes. 100 µl aliquots of the supernatant were inoculated into three wells (cholic acid, deoxycholic acid, control) of a clear bottom black walled 96-well plate, coated with 4T1-RedLuc cells from a mouse cell line that are transformed with luciferase to detect the presence of luciferin. The 96 well plate was read after 20 minutes using an IVIS-100 imaging system and luminescence levels were detected using Living Image software (PerkinElmer).

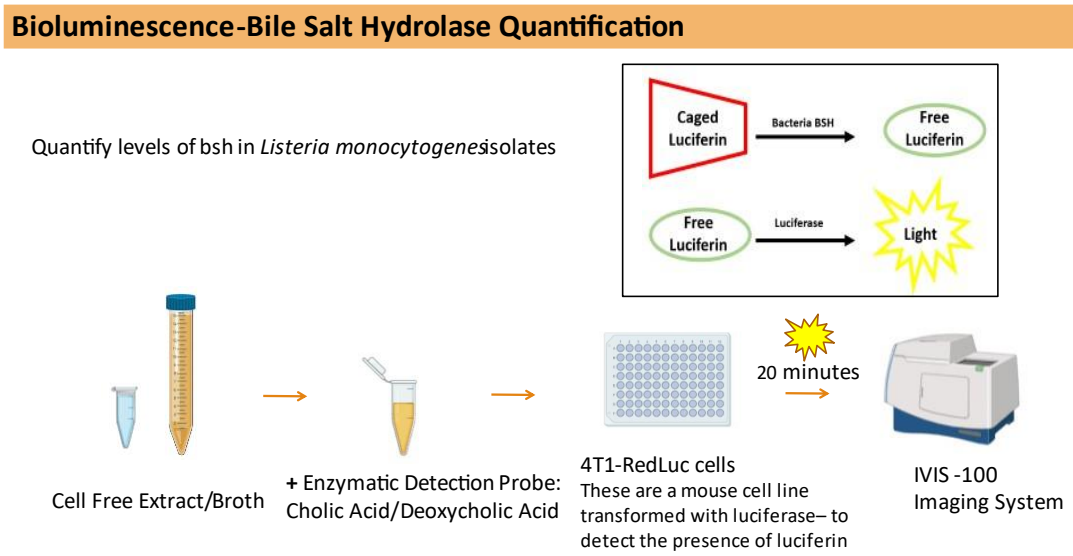


Figure 12. Bile Salt Hydrolase Bioluminescence Probe Assay Schematic. Cell free extracts were mixed with enzymatic detection probes and incubated at 37 °C for one hour. Samples were inoculated into 96 well plates coated with 4T1-RedLuc cells. Luminescence levels, equating to BSH activity, were read using an imaging system.

Bile Salt Hydrolase Polymerase Chain Reaction-Detection

DNA Extraction

A colony of *L. monocytogenes* was added to 1 ml molecular grade water in a 1.5 ml microtube, vortexed and placed in a heating block at 100 °C for 10 minutes, mixing periodically. The sample was allowed to cool before centrifuging at 5,000 g for 5 minutes. The supernatant was transferred to a new microtube and used immediately or stored at -20 °C.

PCR

A PCR master mix was prepared as follows:

For a 50 µl reaction

- 25 µl Speedy Nzy Taq 2 X Green Master Mix (Nzytech MB36202)
- 2.5 µl *bsh* Forward Primer (5' GGTATTGCTGCTGTGATGG 3') Melting Temperature (Tm) 61.8 °C
- 2.5 µl *bsh* Reverse Primer (5' TGAGAAATTGAGTCCTGCCATA 3') Tm 63.4 °C
- 18 µl molecular grade water (DNase/RNase free)
- 2 µl DNA template

Reagents were thawed and kept on ice. The PCR master mix was prepared and 48 µl per sample was aliquoted into 0.2 ml microtubes. 2 µl of DNA template was added to the master mix. *L. monocytogenes* EGDe was used as a *bsh* positive control, *L. innocua* was used as a *bsh* negative control. Molecular grade water was used as the no template control. The samples were placed in a thermal cycler (AppliedBiosystems SimpliAmp) with the following cycling conditions:

- 95 °C for 1 min
- 94 °C for 2 seconds
- 56.8 °C for 5 seconds
- 72 °C for 1 second
- 72 °C for 2 minutes

30 Cycles

- 4 °C for infinity

After the PCR cycles were complete, products were run on a 1.5 % agarose gel (100 ml TAE buffer and 1.5 g agarose) stained with SYBR Safe (4 µl). 10 µl of PCR product was run for each sample. The size of the *bsh* gene target = 87 base pairs, annealing temperature = 56.8 °C. A DNA ladder was run on each side of the gel (NZY DNA Ladder III). The gel was run at 100 V for 3 hours. Bands were visualised using a UV transilluminator.

Bile Salt Hydrolase Reverse Transcription PCR-Quantification

RNA Extraction

Cultures were mixed with RNA later (Sigma Aldrich) and vortexed at room temperature for five minutes. A Qiagen RNeasy Kit was used to extract RNA from samples. To disrupt cells, 600 µl of RLT buffer-B-mercaptoethanol solution was added to each sample and vortexed. A FAST prep-machine was used to break cells (max speed 6000 g for 45 seconds). Samples were cooled on ice for 5 minutes between cycles. This was repeated 3 times followed by centrifugation at 14,000 g for 2 minutes at 4°C. The supernatant was transferred to a new microtube. Equal volumes of 70% ethanol were added and mixed. 700 µl of the mix was added to a spin column and collection tube. The spin columns were centrifuged at 10,000 g for 15 seconds. The flow-through in the collection tube was discarded. 350 µl of RW1 buffer was added to the spin column and centrifuged at 10,000 g for 15 seconds The flow-through in the collection tube was discarded. Using a RNAse- free DNAse kit (Qiagen), 550 µl of RNAse free water was inoculated into a DNAse vial with a syringe and mixed gently. 10 µl of DNAse stock solution was added for every 70 µl of RDD buffer and mixed gently. 80 µl of the mix per RNase spin column was added and incubated at 20-30 °C for 15 minutes. After incubating, 350 µl of RW1 buffer was added to each spin column. Samples were centrifuged at 10,000 g for 15 seconds. The flow-through in the collection tube was discarded. 500 µl of RPE buffer was added and centrifuged for 15 seconds. The flow-through in the collection tube was discarded. Another 500 µl of RPE buffer was added and centrifuged at 10,000 g for 2 minutes. Spin columns were placed into new collection tubes and centrifuged at 10, 000 g for 1 minute. The spin columns were placed into RNAse-free 1.5 ml microtubes. To elute, 50 µl of RNAse-free water was added to each sample and centrifuged at 10,000 g for 1 minute. RNA samples were stored at -80 °C until required.

DNAse Treatment

To remove any contaminating DNA from the RNA samples, a Turbo DNA-free Kit (Invitrogen) was used. RNA samples were thawed on ice. Once thawed, 0.1 volumes of 10 X TURbO DNase buffer and 1 µl TURbO DNase was added to each sample and mixed gently. Samples were incubated at 37 °C for 20-30 minutes. The DNase inactivation reagent was resuspended and 0.1 volumes were added to each sample and mixed well. Samples were incubated for 5 minutes at room temperature and mixed periodically. Samples were centrifuged at 10,000 g for 90 seconds. The supernatant (containing the RNA) was transferred to a new microtube.

Complimentary DNA (cDNA) Synthesis

A NZY First-Strand cDNA Synthesis Kit (Nzytech) was used to synthesis cDNA. Microtubes were kept on ice and the following was added: 10 µl NZYRT 2 X Master mix, 2 µl NZYRT enzyme mix, RNA samples and DEPC-treated water. Samples were mixed and incubated at 25 °C for 10 minutes followed by incubation at 50 °C for 30 minutes and the reaction was inactivated by heating at 85 °C for 5 minutes. Samples were then cooled on ice. 1 µl of NzY RNase H was added to each sample and incubated at 37 °C for 20 minutes. cDNA products were stored at -20°C until required.

Quantitative PCR

To quantify levels of the *bsh* gene in samples. , a master mix was prepared using SYBR Green Supermix, forward and reverse primers (*bsh* primers listed in the detection PCR methodology) and molecular grade water. For each sample, 10 µl of the master mix was aliquoted (in triplicate) into a 96 well plate and the cDNA template was added. The 16S (housekeeping gene) acted as a control for the experiment: 16S Forward Primer (5’ TGGAAACTGGAGAAGACTGG 3’), 16S Reverse Primer (5’ GTTCCTCCACATATCTACG 3’). Samples were analysed by Real-Time PCR (Figure 13).

Amplification curves and CT values were analysed to quantify *bsh* levels in the samples.

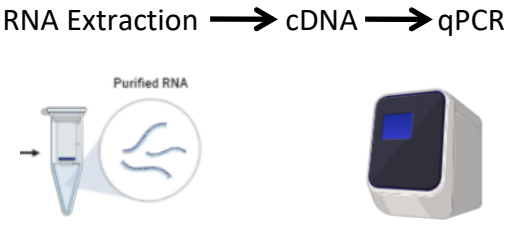


Figure 13. Reverse Transcription Quantitative PCR. RNA was extracted from samples and reverse transcribed into cDNA. Samples were analysed by Real-Time PCR to quantify *bsh* levels.

Results

Bile Resistance Assay

Development Work

As there was a large bank of *L. monocytogenes* isolates to screen for bile resistance, the different parameters including the selection of bile, bile concentration, bile exposure time, growth medium/pH and environmental conditions were assessed, using a selection of isolates, to develop an appropriate and reproducible assay to screen all 228 isolates. Two isolates, control strain EDGe and sample 966 were used to trial: bile concentration, growth medium/pH, growth environment and bile exposure time. Results were reviewed and a larger assortment of isolates (944, 948, 950, 960, 987, 1101) were trialled for the bile selection using different concentrations of porcine bile incorporating two pH values, pH 7.5 and pH 5.5. Results are displayed as "Log CFU/ml" or "percentage bile resistance" with the term "bile resistance" implying the percentage of viable cells remaining after bile exposure.

Selection of Bile

Dried purified ox-bile, TDCA-a bile acid and dried porcine bile extract were assessed for suitability. The isolates were exposed to a bile/bile acid broth for 1 hour using standard environmental conditions. The different types of bile caused a reduction in bacteria to varying degrees. TDCA worked well in the assay but due to the large quantities of the expensive bile acid required to test all 228 isolates in triplicate, it was not viable. Ox-bile and porcine bile worked well with the assay but higher concentrations of the ox-bile were required for an inhibitory effect. Porcine bile is most similar to human bile in terms of chemical constituents and is more inhibitory than bovine bile at lower concentrations. For both porcine and ox-bile, higher concentrations (5% and 10%) resulted in solubility issues and inconsistent results. After reviewing results, dried porcine bile extract was selected for the assay. Using preliminary results (not shown), three isolates exhibiting a lower tolerance to bile (948, 960, 987) and three isolates exhibiting a higher tolerance to bile (944, 950,

1101) were screened using 1%, 1.5% and 2% porcine bile at pH 7.5 and pH 5.5 (Figure 14) to assess optimum assay conditions.

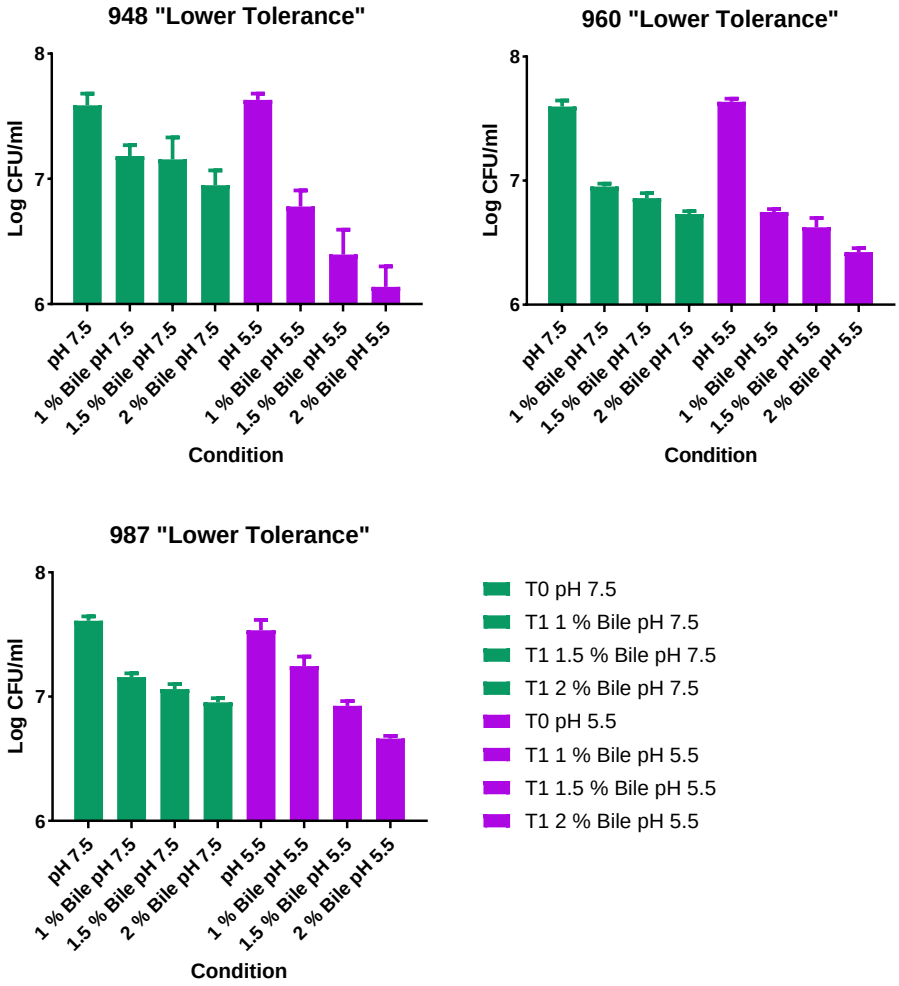


Figure 14 (A)

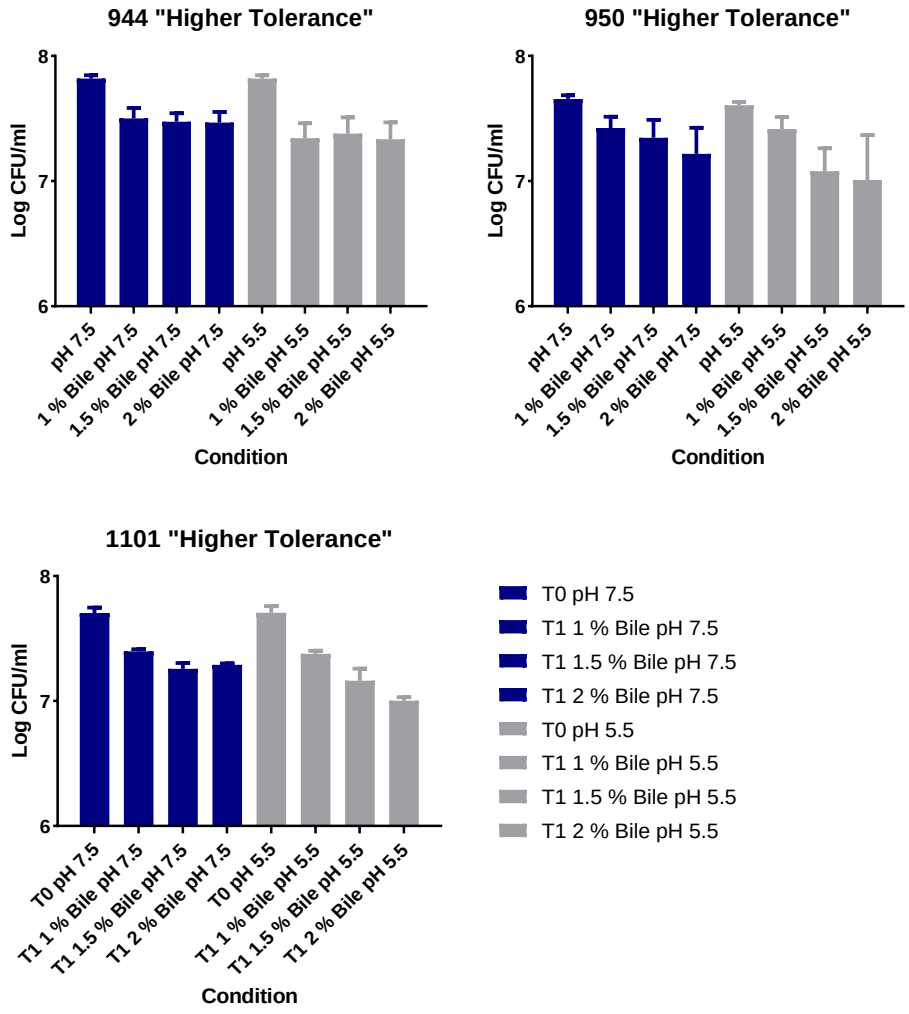


Figure 14 (B)

Figure 14. Selection of Bile. The suitability of porcine bile was assessed using a selection of *L. monocytogenes* isolates. Preliminary results (not shown) were reviewed and three cultures exhibiting a "lower tolerance" to bile (Figure 14A: 948, 960, 987) and three cultures exhibiting a "higher tolerance" to bile (Figure 14B: 944, 950, 1101) were selected for testing. Different bile concentrations and pH conditions were incorporated. Overnight cultures were inoculated into BHI broth pH 7.5/pH 5.5 or BHI pH 7.5/5.5 bile broth. Viable plate counts were performed using a BHI broth without bile (T0) and after one hour (T1) at 37 °C for BHI bile broths (1%, 1.5%, 2% bile). Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Plates were incubated at 37 °C for 18 to 24 hours. The Log CFU/ml was calculated for each sample. Comparison of the Log CFU/ml for the T0 pH 7.5/5.5 value with the T1 bile broth values (1%, 1.5%, 2%) demonstrated the effect of bile on viability (Figure

14A, Figure 14B). Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

Bile Concentration

Varying concentrations of bile were assessed for suitability. Isolates were exposed to a bile broth for 1 hour using standard environmental conditions. There were solubility issues for bile types tested at higher concentrations (5% and 10%) resulting in inconsistent results and larger error bars, for some of the samples. The higher the concentration, the more difficult to solubilise and remain in solution. Higher concentrations were included to determine the solubility potential of each bile type and were greater than the equivalent human bile levels. Dried porcine bile extract was selected to screen all isolates due to its similarity to human bile and the ability to use a lower concentration in comparison with the other bile types. To assess the solubility potential of the bile, a range of porcine bile concentrations were tested: 1%, 2%, 3%, 4%, 5%, 10%. Concentrations of up to 4% resulted in incremental reductions in bile resistance for the EGDe and 966 isolates tested (Figure 15). A concentration of 1% porcine bile was selected to screen all isolates. At this concentration the bile solubilised well and equated to the average concentration of bile in the human gut.

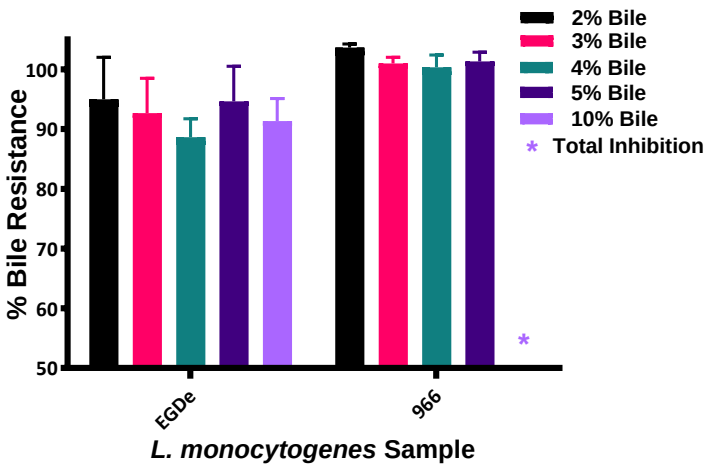


Figure 15. Effect of Concentration on Bile Resistance. Overnight cultures were inoculated into different concentrations of porcine bile BHI broth: 2%, 3%, 4%, 5%,

10%, to assess the effect of concentration on bile resistance. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) for the different concentration BHI bile broths. Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were used to calculate bile resistance. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

Growth Medium/pH

BHI broth was selected for the assay as it is routinely used to cultivate *L. monocytogenes*. Two different pH conditions were tested: pH 7.5 and pH 5.5. Standard environmental conditions were used. Results for a selection of isolates demonstrated a decrease in bile resistance at the lower pH in comparison with the higher pH. *L. monocytogenes* EGDe tolerance results were similar at both pH values. A decrease in bile resistance was observed for sample 966 at pH 5.5 (Figure 16). The lower pH, pH 5.5 was selected for the assay as it equates to the average pH value in the human GI tract where *L. monocytogenes* encounters bile.

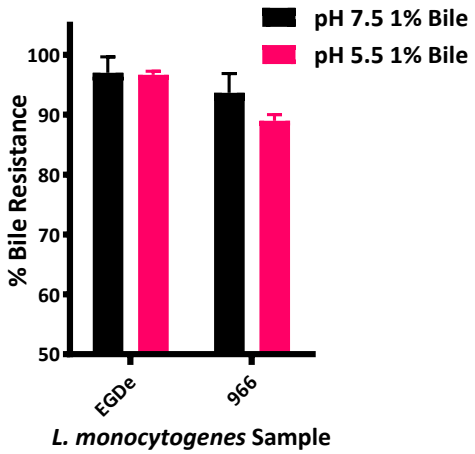


Figure 16. Effect of pH on Bile Resistance. Overnight cultures were inoculated into a BHI bile broth adjusted to two different pH values: pH 7.5 and pH 5.5. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) at 37 °C for the BHI bile broths. Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were

used to calculate bile resistance. The toxicity of bile was observed to increase at a lower pH. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

Growth Environment

Overnight broth cultures were grown aerobically and anaerobically to assess the impact of growth environment on bile resistance. The isolates were exposed to a bile broth for 1 hour. Cultures grown aerobically were more bile tolerant and there was little variation between the different isolates (Figure 17). Cultures grown anaerobically were more affected by bile and generated more interesting bile tolerance profiles. Anaerobic growth conditions were selected to screen all 228 isolates. This environment is most in-line with conditions in the human GI tract, where there is a lowered oxygen availability. In addition, anaerobic conditions are not as well studied as aerobic. For comparison purposes, a proportion of the isolates grown aerobically were tested also (127/228) to further assess the impact of growth environment.

Aerobic: Shaking Incubation
Anaerobic: Gas Jar/Sachet

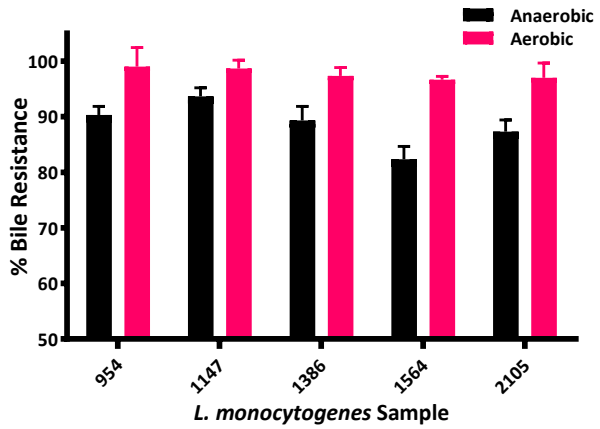


Figure 17. Effect of Environmental Conditions on Bile Resistance. Cultures were grown aerobically or anaerobically for 18 to 24 hours before inoculating into a BHI broth/BHI 1% bile broth. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) at 37 °C for the BHI bile broths. Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were used to calculate bile resistance.

Anaerobic cultures were less tolerant of bile than aerobic cultures. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

Bile Exposure Time

Previous bile tolerance work carried out by Dr. Máire Begley observed an initial kill after one hour exposure to bile (Figure 3). After an extended lag phase, the bacteria began to acclimatise to the environment and numbers levelled off or increased, to varying degrees, over a 10-hour period. One-hour and two-hour bile exposure times were assessed for the assay using bile and bile acid, TDCA. The two-hour trial resulted in total inhibition for some bacteria including EGDe TDCA 5mM, EGDe 1% bile and 966 1% bile (Figure 18). The one-hour exposure demonstrated more variation between the isolates. This time period was selected to provide a "snap-shot" into bile resistance profiles and allow for comparison between the different isolates and groups (food/environmental, clinical).

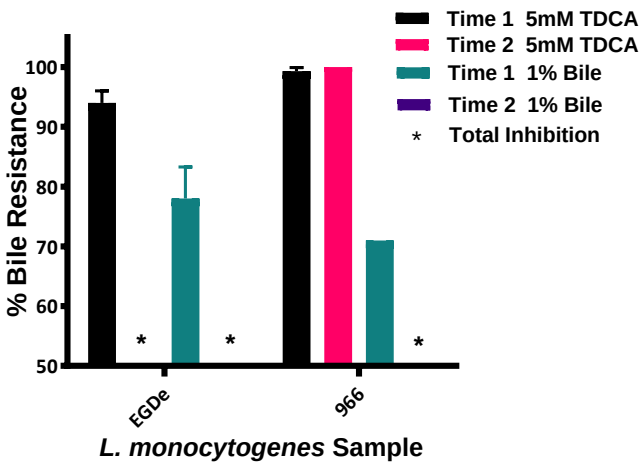


Figure 18. Effect of Bile Exposure Time on Resistance. Overnight cultures were inoculated into a BHI broth/BHI bile broth (TDCA 5mM/1% bile) and incubated at 37 °C for one- and two-hour intervals. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one-hour (T1) or two hours (T2) at 37 °C for the BHI bile broths. Samples were serial diluted in Ringer's solution and enumerated

using BHI agar. Difference between T0 and T1/T2 values were used to calculate bile resistance. The two-hour bile exposure resulted in the total inhibition of growth for some of the isolates tested. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

Developed Bile Resistance Assay-Screen for *L. monocytogenes* Isolates

Anaerobic/Aerobic Results

All 228 *L. monocytogenes* isolates were screened anaerobically for bile resistance. All samples were tested in biological and technical triplicate. For comparison, a selection of food, environmental and clinical isolates were screened aerobically (127/228) to assess the impact of environmental growth conditions on bile resistance. Isolates were grown either anaerobically or aerobically overnight (18 to 24 hours) and exposed to a 1% porcine bile extract pH 5.5 BHI broth for one hour at 37 °C. Aliquots were serially diluted and enumerated using BHI agar. The bile resistance was calculated for each isolate using the difference between Time 0 and Time 1 values (Table 2 and Table 3). Results were graphed and variations in bile resistance were analysed (Figure 19, Figure 20, Figure 21, Figure 22). The isolates grown anaerobically resulted in the greatest variation in the bile resistance profiles and generated the most interesting results (Figure 19, Figure 20).

The isolates grown anaerobically (food/environmental/clinical) had a maximum bile resistance of 100%, a minimum of 55% and an average of 86%. As a large number of samples were screened, results were reviewed to identify isolates with interesting bile tolerance profiles. Isolates of interest include seafood isolate 1388. This isolate was most affected by bile from the food and environmental group with an average result of 56% bile tolerance. This was the lowest recorded result out of all 228 isolates. 1109 and 1317, environmental isolates, had bile resistance profiles of 57% and 61% respectively, 1370 a meat isolate had a resistance of 62% and 1880, a mushroom isolate, had a resistance of 64%. Higher bile tolerance food and

environmental isolates included 1515 and 1798, an environmental isolate and a milk isolate with 100% resistance.

The average bile resistance for the clinical isolates grown anaerobically was 91% with a maximum value of 100% and a minimum value of 72%. From the clinical group, G-041 had the lowest bile tolerance out of the clinical isolates at 72% with 10403S a control strain and MQ150012 a blood isolate resulting in 74% bile resistance. There were two mother and infant pairs represented in the clinical isolates. Results were comparable between the mother and infant; MQ140011 and MQ140012 anaerobic growth bile tolerance of 89% and 91% respectively; MQ140034 and MQ140035 anaerobic growth bile tolerance of 95% and 97% respectively. The clinical isolates resulted in higher bile tolerance profiles when compared to the food and environmental isolates.

In comparison, results for the aerobically grown isolates demonstrated a consistently higher level of bile tolerance across all isolates and between the food/environmental and clinical groups. Only a selection of isolates from the two groups (food/environmental 58/154, clinical 69/74) were screened aerobically as preliminary results (Figure 17) showed little difference between the bile resistance profiles and as a result, the assay focused mainly on the anaerobic growth environment. The selection of isolates tested had similar bile resistance patterns (Figure 21 and 22) with little variation. The cultures remained relatively unaffected by bile with a maximum result of 100%, a minimum of 91% and an average of 98%, for both groups and collectively. Isolates of interest include F2170/17, an environmental isolate, which had the lowest bile resistance at 92% and clinical isolate, G-011, recorded the lowest resistance of the clinical isolates at 91%. Results from the aerobic assay did not demonstrate significant differences in bile tolerance profiles.

Result comparison between the anaerobic and aerobic assay demonstrated that isolates grown anaerobically were more affected by bile and were less tolerant than cultures grown aerobically. Results indicate that environmental growth conditions have the potential to influence bile resistance. Results from the anaerobically grown

isolates were selected to study further. Results were analysed by lineage and clonal complex to establish if there were bile tolerance trends or patterns. An unpaired t-test (two-tailed), was performed on the results for the isolates grown anaerobically to compare the means of the food/environmental samples and clinical samples for bile resistance. A value of ≤ 0.05 was considered statistically significant in each test. Interestingly, there was a significant difference between the means of the two data sets: p value = < 0.001, indicating that the clinical isolates are statistically more resistant to bile than the food and environmental isolates. A non-parametric Mann-Whitney test also determined statistical significance (p value = < 0.001). This is an important finding and highlights the evolutionary pressure on these strains to survive in the gut. Statistical analysis, completed on results from the aerobically grown isolates, generated a p value = 0.40 signifying no significant difference between the two groups.

Results by Lineage

Anaerobic and aerobic results were grouped by lineage to assess if there is any link between lineage and bile resistance (Figure 23, Figure 24). As the had similar bile resistance patterns, results were not analysed further. Results from the anaerobically grown isolates were selected to study. The majority of the isolates had lineage information available (215/228). Isolates were grouped into lineage I or lineage II. 99 isolates were identified as lineage I of which 61 were food or environmental isolates and 38 were clinical. 116 isolates were identified as lineage II of which 88 were food or environmental isolates and 28 were clinical. An unpaired t-test compared the means of the sample results from Lineage I and Lineage II. There was no significant statistical difference between the lineages (p value = 0.19). A link between bile resistance and lineage was not established.

Results by Clonal Complex

Results from the anaerobic assay were used to compare isolates by clonal complex to identify any potential patterns relating to bile resistance (Figure 25). Clonal complex information was available for 210/228 of the isolates. CC121 represented the largest group with 43 isolates comprising of 38 food/environmental and 5 clinical

isolates. The bank of samples included a range of difference clonal complexes including:

- CC1 (20 isolates-5 food/environmental, 15 clinical)
- CC2 (18 isolates-12 food/env, 6 clinical)
- CC3 (4 isolates-3 food/env, 1 clinical)
- CC4 (3 isolates-2 food/env, 1 clinical)
- CC5 (14 isolates, 13 food/env, 1 clinical)
- CC6 (9 isolates-2 food/env, 7 clinical)
- CC7 (7 isolates-5 food/env, 2 clinical)
- CC8 (6 isolates-3 food/env, 3 clinical)
- CC9 (7 isolates-6 food/env, 1 clinical)
- CC14 (4 isolates-3 food/env, 1 clinical)
- CC18 (7 isolates-5 food/env, 2 clinical)
- CC20 (5 isolates-4 food/env, 1 clinical)
- CC26 (1 food/env isolate)
- CC31 (2 food/env isolates)
- CC37 (10 isolates-6 food/env, 4 clinical)
- CC54 (10 isolates-6 food/env, 4 clinical)
- CC59 (7 isolates, 6 food/env isolates, 1 clinical)
- CC87 (2 isolates, 1 food/env, 1 clinical)
- CC90 (2 clinical isolates)
- CC101 (5 isolates-1 food/env, 4 clinical)
- CC121 (43 isolates-38 food/env, 5 clinical)
- CC155 (7 isolates-6 food/env, 1 clinical)
- CC204 (2 food/env isolates)
- CC220 (8 isolates-7 food/env, 1 clinical)
- CC224 (1 food/env isolate)
- CC226 (1 clinical isolate)
- CC379 (1 food/env isolate)
- CC412 (1 food/env isolate)

- CC475 (1 clinical isolate)
- CC787 (2 food/env isolates)

Similar to the findings grouped by lineage, variations in bile resistance were observed between the different CCs and isolates from within the same CC. Two food/environmental isolates (1388 and 1317), that had the lowest bile resistance in the study, were grouped into CC2, commonly associated with clinical listeriosis cases. Other isolates in CC2 had higher bile tolerance profiles with an overall average of 81% collectively. When analysed by group, the CC2 food and environmental isolates had an average bile resistance of 78%, whereas, the CC2 clinical strains had an average resistance of 89%, demonstrating higher levels of tolerance.

Two CCs of interest include CC1, commonly associated with clinical cases, which had an overall higher level of bile resistance at 90% and CC18 which had the highest average bile resistance at 96% when compared with the other CCs (Figure 26). 4/5 of the CC18 isolates (food/environmental) were over 96% resistant with the clinical isolates (2) having an average resistance at 93%. CC121 the largest group, had variations in bile resistance across all isolates. Just under half of the isolates (19) had a bile tolerance below 80% and the other half (24), including five clinical strains, had a higher level of resistance over 80%. The CC121 food and environmental isolates reported varied bile resistance ranging from 77% to 100%. In comparison the clinical isolates demonstrated higher patterns of bile resistance at over 95%.

Bile resistance, overall, varied by CC. To complete a more detailed analysis on potential associations between CC and bile resistance, additional isolates are required with equally represented CC group numbers.

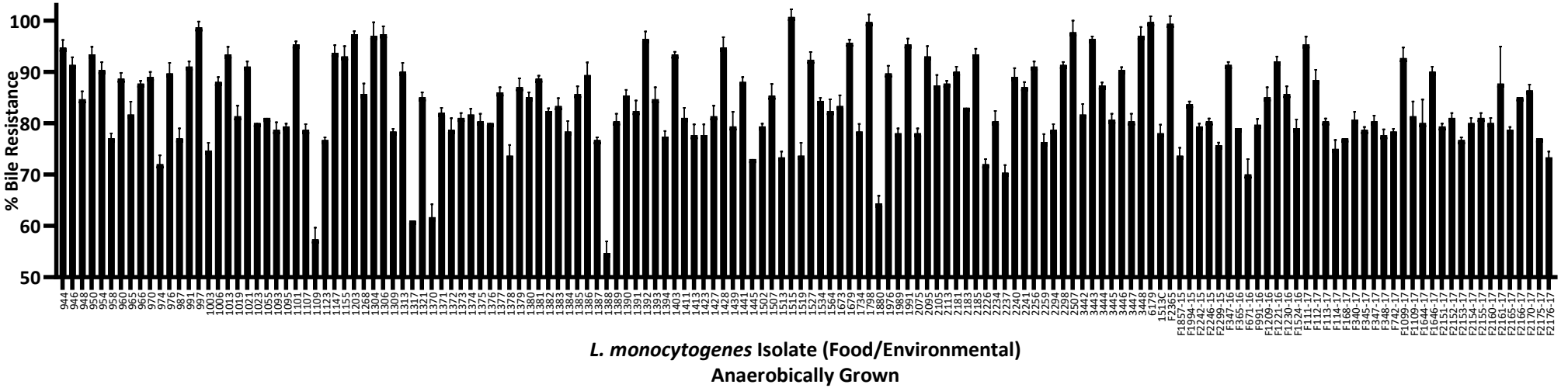


Figure 19. **Food/Environmental** *L. monocytogenes* isolates were grown **anaerobically** in BHI broth at 37 °C for 18 to 24 hours. Overnight cultures were inoculated into pH 5.5 BHI broth or pH 5.5 BHI broth supplemented with 1% porcine bile and incubated for 1 hour at 37 °C. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) for the bile broth. Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were used to calculate bile resistance. Results demonstrated a high level of variation in the bile resistance profiles with a selection of higher and lower tolerant isolates. Isolate 1388 reported

the lowest bile resistant result with a mean value of 56%. Group mean 83.60% +/- SD 8.40. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

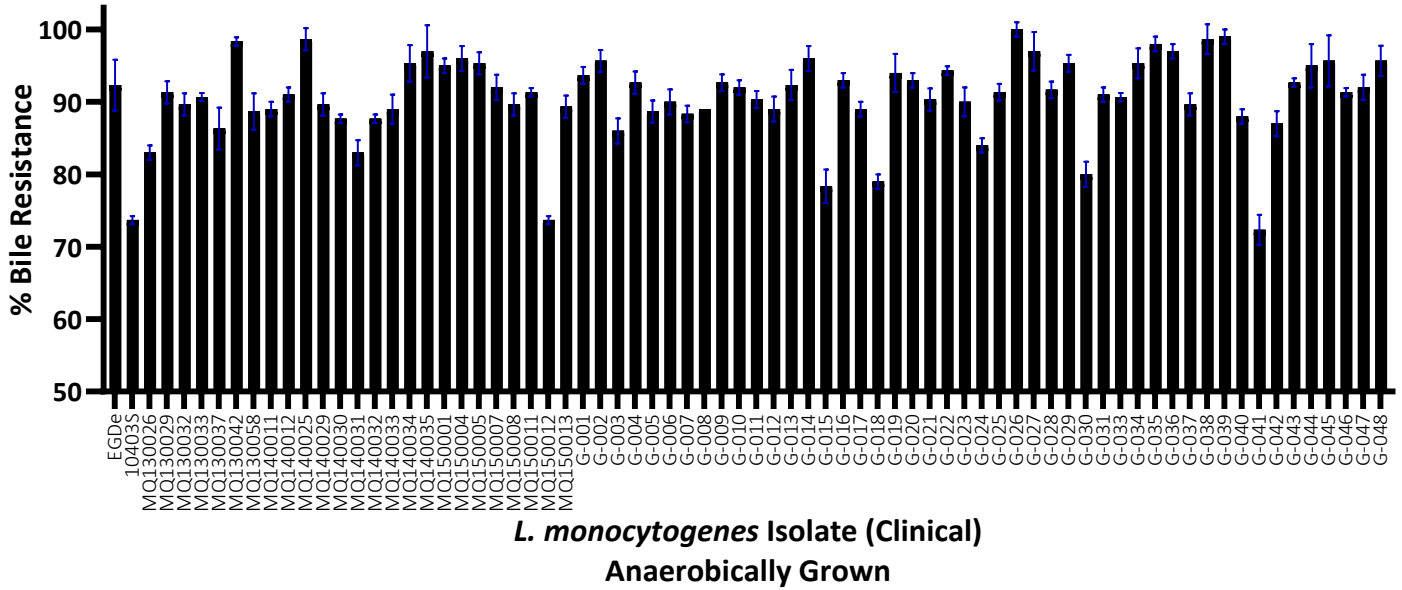


Figure 20. **Clinical** *L. monocytogenes* isolates were grown **anaerobically** in BHI broth at 37 °C for 18 to 24 hours. Overnight cultures were inoculated into pH 5.5 BHI broth or pH 5.5 BHI broth supplemented with 1% porcine bile and incubated for 1 hour at 37 °C. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) for the bile broth. Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were used to calculate bile resistance. Results demonstrated a

variation in the bile resistance profiles with a higher level of tolerance when compared with the food and environmental group. Group mean 90.68% +/- SD 5.83. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

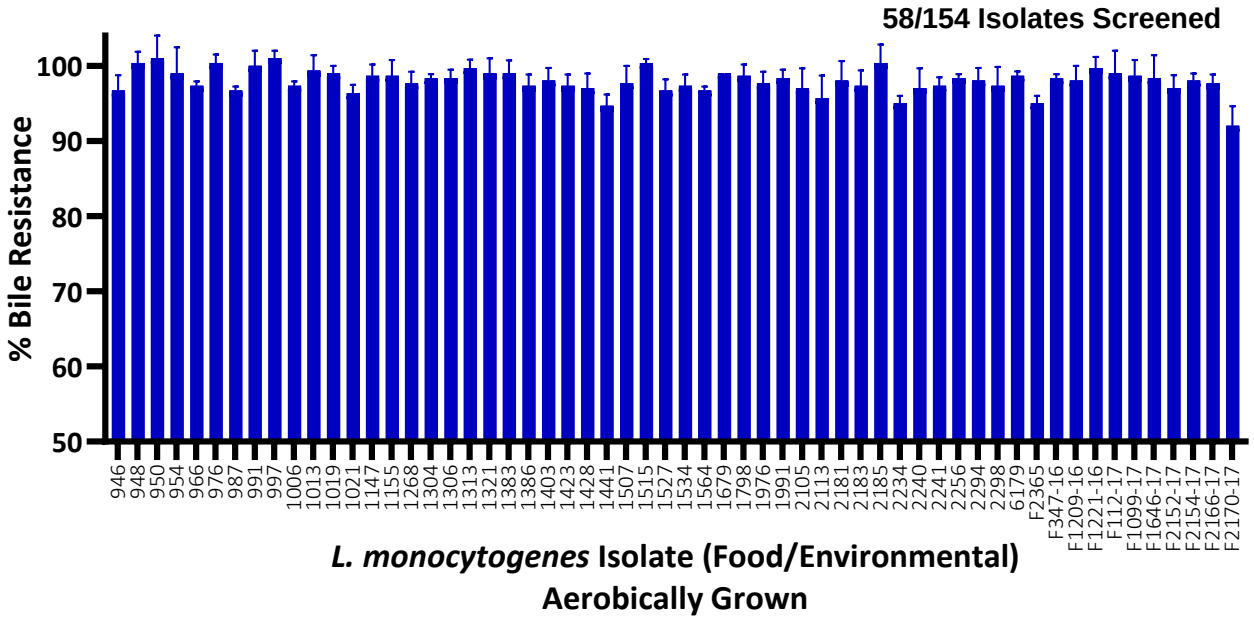


Figure 21**. Food/Environmental** *L. monocytogenes* isolates (selection 58/154) were grown **aerobically** in BHI broth at 37 °C for 18 to 24 hours. Overnight cultures were inoculated into pH 5.5 BHI broth or pH 5.5 BHI broth supplemented with 1% porcine bile and incubated for 1 hour at 37 °C. Viable plate counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) for the bile broth. Samples were

serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were used to calculate bile resistance. Results indicated a high level of bile resistance with little variation. Group mean 97.98% +/- SD 1.61. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

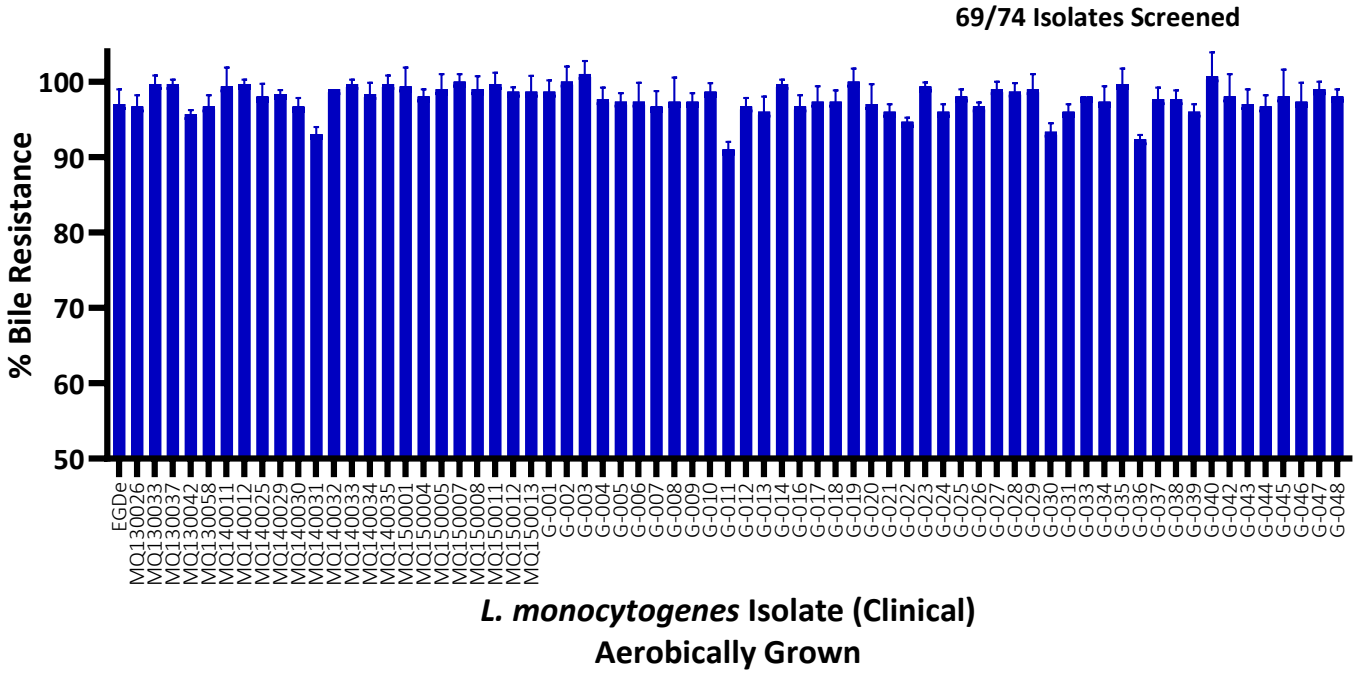


Figure 22. **Clinical** *L. monocytogenes* isolates (selection 69/74) were grown **aerobically** in BHI broth at 37 °C for 18 to 24 hours. Overnight cultures were inoculated into pH 5.5 BHI broth or pH 5.5 BHI broth supplemented with 1% porcine bile and incubated for 1 hour at 37 °C. Viable plate

counts were performed at time 0 (T0) using a BHI broth without bile and after one hour (T1) for the bile broth. Samples were serial diluted in Ringer's solution and enumerated using BHI agar. Difference between T0 and T1 values were used to calculate bile resistance. Results indicated a high level of bile resistance with little variation. Group mean 97.71% +/- SD 1.92. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

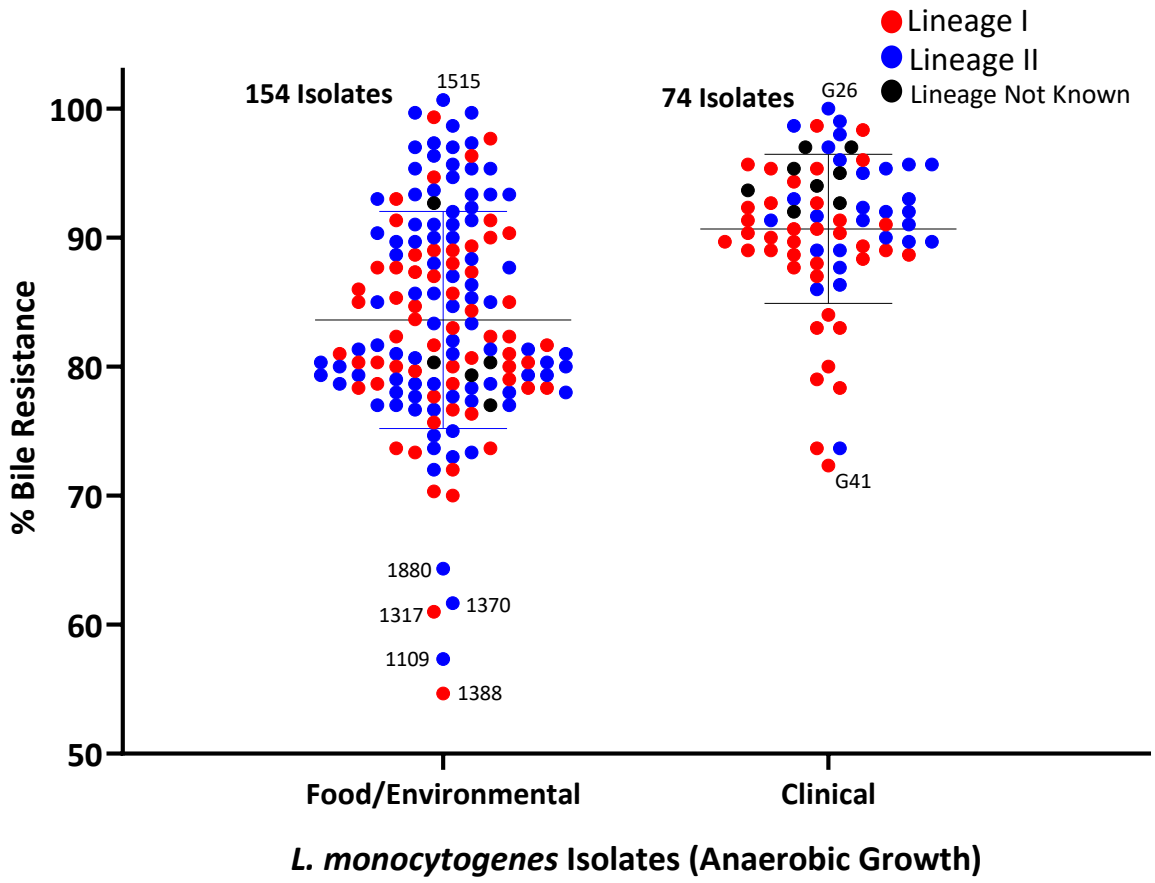


Figure 23. Bile resistance results for **anaerobically** grown isolates were grouped by **lineage** and **sample origin** (food/environmental versus clinical). Red represents lineage I and blue represents lineage II strains. Black represents strains with unknown lineage. Cultures grown anaerobically resulted in more varied bile resistance profiles than the cultures grown aerobically. Clinical isolates resulted in statistically higher bile resistance than the food and environmental isolates (p value = < 0.001). Statistical significance was determined by unpaired t test. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

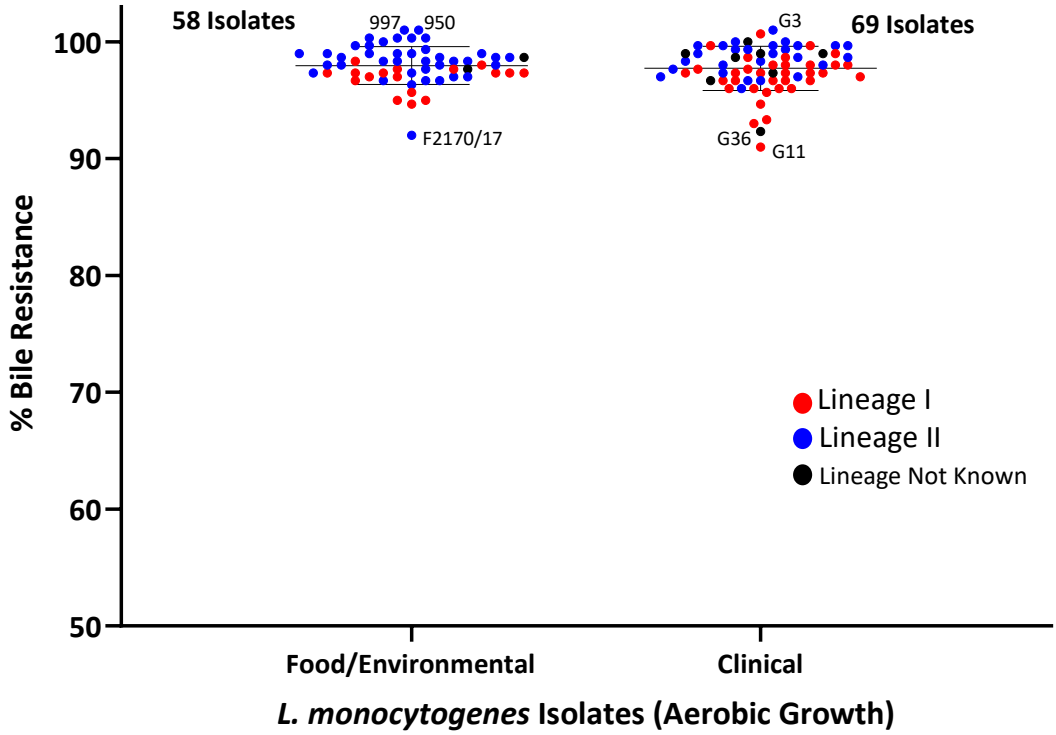


Figure 24. Bile resistance results for **aerobically** grown isolates were grouped by **lineage** and **sample origin** (food/environmental versus clinical). Red represents lineage I and blue represents lineage II strains. Black represents strains with unknown lineage. Cultures grown aerobically resulted in higher levels of bile resistance across all isolates tested. There was no differentiation between food/environmental and clinical groups. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

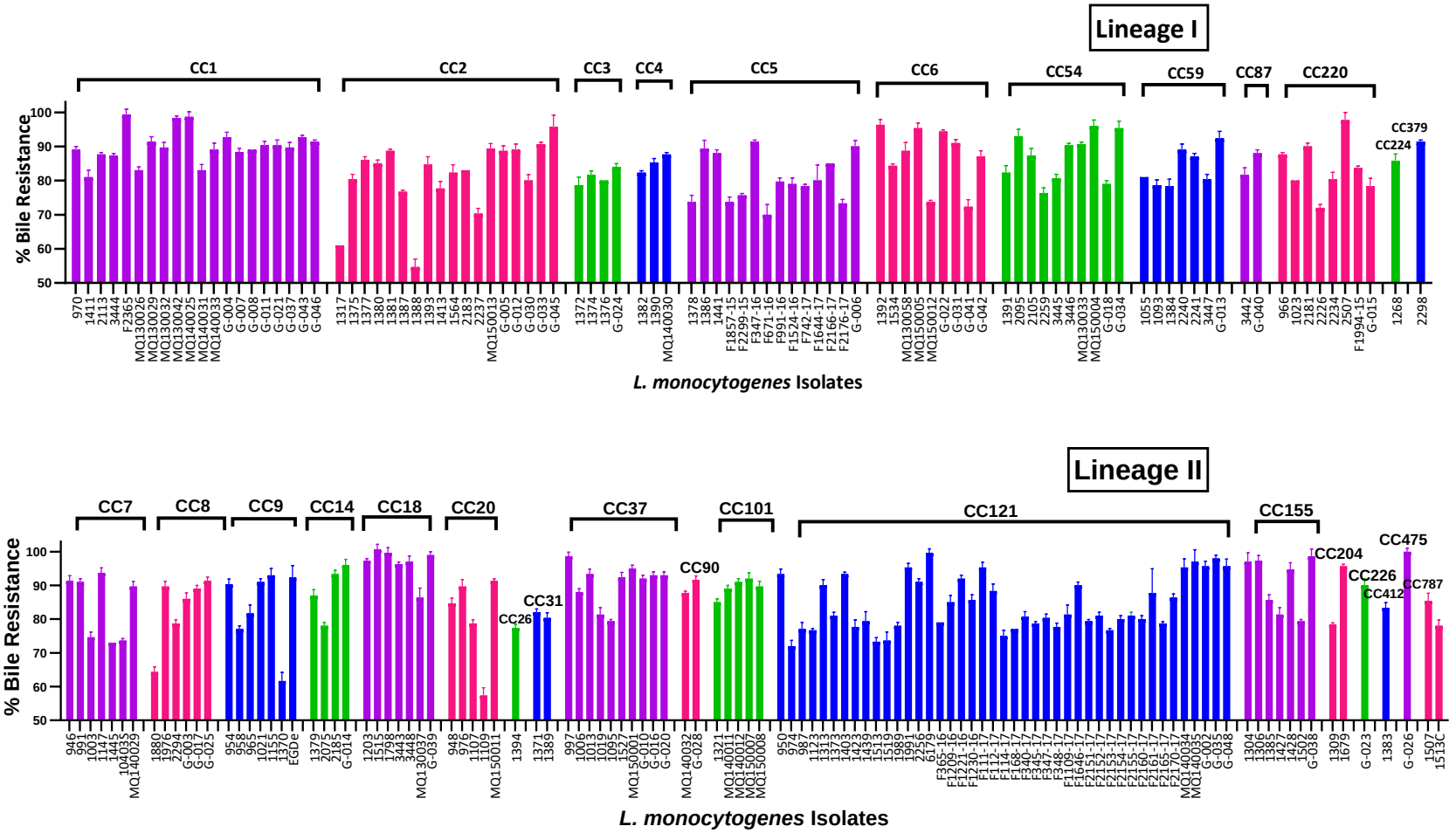

Lineage I
CC1
CC2
CC3
CC4
CC5
CC6
CC54
CC59
CC87
CC220
CC379
CC224
% Bile Resistance
100
90
80
70
60
50
970
1411
2113
3444
F2365
MQ130026
MQ130029
MQ130032
MQ130042
MQ140025
MQ140031
MQ140033
G-004
G-007
G-008
G-011
G-021
G-037
G-043
G-046
1317
1375
1377
1380
1381
1387
1388
1393
1413
1564
2183
2237
MQ150013
G-005
G-012
G-030
G-033
G-045
1372
1374
1376
G-024
1382
1390
MQ140030
1378
1386
1441
F1857-15
F2299-15
F347-16
F671-16
F991-16
F1524-16
F744-17
F1644-17
F2166-17
F2176-17
G-006
1392
1534
MQ130058
MQ150005
MQ150012
G-022
G-031
G-041
G-042
1391
2095
2105
2259
3445
3446
MQ130033
MQ150004
G-018
G-034
1055
1093
1384
2240
2241
3447
G-013
3442
G-040
966
1023
2181
2226
2234
2507
F1994-15
G-015
1268
2298
L. monocytogenes Isolates
Lineage II
CC7
CC8
CC9
CC14
CC18
CC20
CC37
CC101
CC121
CC155
CC475
CC204
CC226
CC412
CC787
CC90
CC31
CC26
% Bile Resistance
100
90
80
70
60
50
946
991
1003
1147
1445
10403S
MQ140029
1880
1976
2294
G-003
G-017
G-025
954
958
965
1021
1155
1370
EGDe
1379
2075
2185
G-014
1203
1578
1798
3443
3448
MQ130037
G-039
948
976
1109
MQ150011
1394
1371
1389
997
1006
1013
1019
1025
1035
MQ150001
G-010
G-016
G-020
MQ140032
G-028
1321
MQ140011
MQ140012
MQ150007
MQ150008
950
974
987
1123
1313
1373
1403
1433
1513
1519
1989
1991
2256
6179
F3265-16
F1209-16
F1221-16
F1230-16
F111-17
F112-17
F114-17
F166-17
F340-17
F345-17
F347-17
F348-17
F1109-17
F1646-17
F2151-17
F2153-17
F2154-17
F2155-17
F2160-17
F2161-17
F2165-17
F2170-17
MQ140034
MQ140035
G-002
G-035
G-048
1304
1305
1427
1428
1502
G-038
1309
1679
G-023
1383
G-026
1507
1513C
L. monocytogenes Isolates

Figure 25. Bile resistance results for **anaerobically** grown isolates were grouped by **clonal complex** group to identify any potential patterns relating to bile resistance. Bile resistance varied between the different CCs and amongst individual CC groups. Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

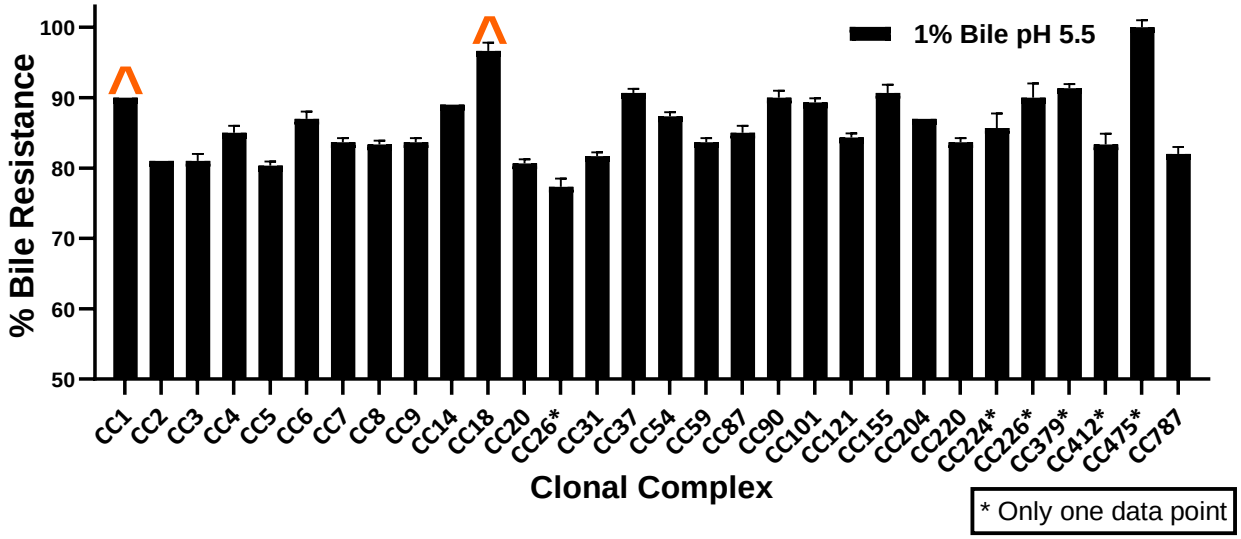


Figure 26. The average anaerobic bile resistance value for each CC was grouped to identify any potential patterns relating to bile resistance. Isolates marked with a * only had one isolate included in the CC. CC1 and CC18, indicated by ^ demonstrated a higher level of bile resistance compared to other CC groups (CC475 only one isolate for comparison). Samples were tested in biological and technical triplicate. The error bars represent the mean +/- standard deviation.

Bile Salt Hydrolase Agar Plate Assay

Development Work

Due to the large number of *L. monocytogenes* isolates to screen for bile salt hydrolase activity, the different parameters including the agar type, bile type and environmental conditions were optimised to develop a suitable assay.

Selection of Agar

BHI agar was trialled with porcine bile (0.5% and 1%) and bile acids, GDCA and TDCA (0.5%), but non-specific precipitation was observed. The precipitation was independent of BSH activity and presented as an opaque halo surrounding the culture inoculum. BHI agar was not suitable for the assay. MRS agar is used for Lactobacillus growth. MRS agar will not support the growth of Listeria but it will facilitate the BSH reaction. The procedure involved inoculating the agar surface with *L. monocytogenes* cultures grown on BHI agar. The method facilitates the reaction between bile acids in the agar and BSH positive cultures, presenting as white zones of precipitation surrounding the inoculum. MRS agar contains Tween and this surfactant may have a role in facilitating the precipitation reaction. Oxoid MRS agar (Tween included as an ingredient) was trialled but precipitation was poor. A different brand of MRS was sourced from Merck, which included the addition of Tween 80 prior to autoclaving. The agar was trialled and worked very well to detect BSH activity in the *L. monocytogenes* culture (Figure 28). Different inoculation methods were trialled using broth and agar plate cultures. The broth cultures didn't work well and resulted in very weak reactions. Patch inoculating agar plate cultures onto the bile plates worked best and was used to screen the bank of isolates (Figure 27).



Figure 27. Bile plate inoculation. *L. monocytogenes* patch inoculation of MRS agar supplemented with 0.5% GDCA/TDCA (wt/vol) to detect/quantify BSH activity.

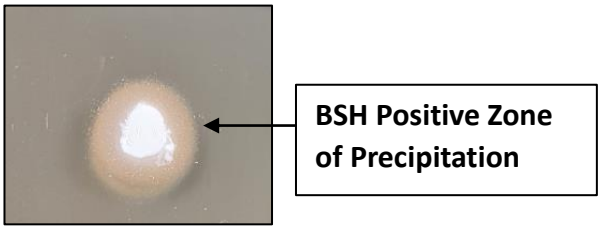


Figure 28. BSH activity of *L. monocytogenes* EGDe. Cultures were patch inoculated onto MRS agar supplemented with 0.5% GDCA/TDCA and incubated anaerobically. BSH activity presented as white precipitate halos of unconjugated bile acids after 48/72 hours anaerobic incubation.

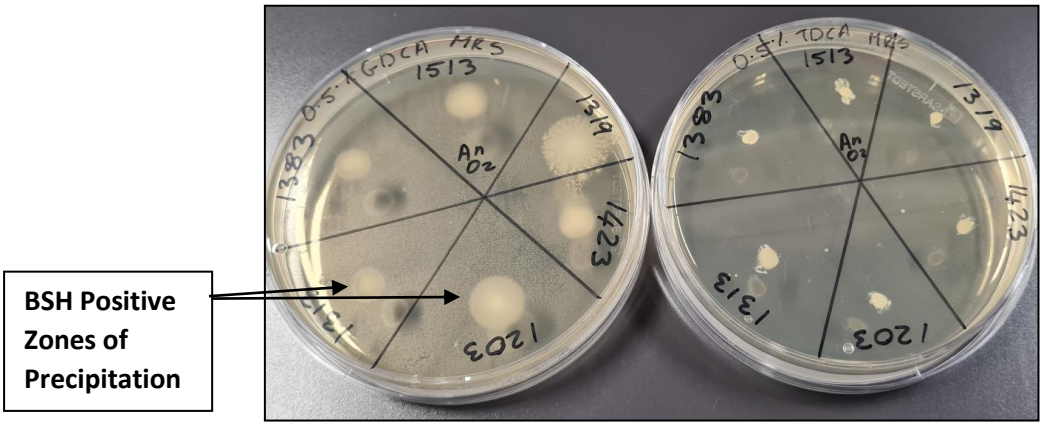


Figure 29. BSH activity after 48 hours anaerobic incubation on 0.5% GDCA MRS Agar (left) and 0.5% TDCA MRS Agar (right). GDCA produced more positive and definitive zones of precipitation than TDCA.

Bile Type

Porcine Bile (0.5% and 1%) was initially trialled but non-specific precipitation resulted (Figure 30). This precipitation was independent of BSH activity and may have been a reaction between the porcine bile and the agar medium. The negative control (*L. innocua*) resulted in a positive reaction presenting as an opaque halo around the inoculum.

Purified bile acids, GDCA and TDCA were trialled and worked best to detect BSH activity (Figure 29). Due to the expense of the purified bile acids and the large quantity of isolates to screen, a concentration of 0.5% bile acid (wt/vol) was selected for the assay. This is the concentration used in the original assay developed by Dashkevicz and Feighner.

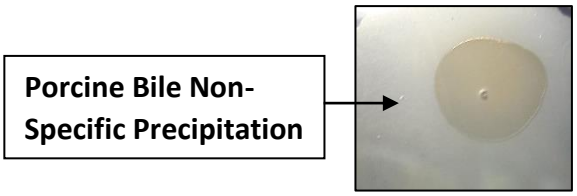


Figure 30. Porcine bile non-specific precipitation. Overnight broth cultures were inoculated onto BHI agar supplemented with 0.5% porcine bile and incubated anaerobically for 48 to 72 hours. Non-specific opaque halos resulted for all cultures including the BSH negative control (*L. innocua*). Similar results were recorded for patch inoculated bile plates (not shown).

Environmental Conditions

Trials were completed using both aerobic and anaerobic growth conditions to assess the effect of environment. For anaerobic conditions, bile agar plates were stored in an anaerobic jar with a gas sachet at room temperature for at least 72 hours before use. Once inoculated, the agar plates were incubated in anaerobic conditions at 37 °C for 72 hours. Aerobic plates were stored and incubated in standard environmental conditions. The plates were read at 48 hours and 72 hours to determine BSH activity. The plates incubated anaerobically had slightly larger zones of precipitation in

comparison to aerobic plates. As the bile resistance response may be triggered by an anaerobic environment, all isolates were screened anaerobically.

Developed Bile Salt Hydrolase Agar Plate Assay-Screening System for *L. monocytogenes* Isolates

All 228 *L. monocytogenes* isolates were assessed for levels of bile salt hydrolase activity. Cultures were inoculated onto MRS agar supplemented with bile acids GDCA or TDCA at a concentration of 0.5% (wt/vol). Plates were stored in an anaerobic jar for at least 72 hours before use. Inoculated plates were incubated anaerobically at 37 °C for 72 hours and read after 48 and 72 hours. Due to the numbers of isolates and the expense of the bile acids, samples were tested in duplicate only. BSH activity presented as a white zone of unconjugated bile acid precipitation. Diameter zone size was measured in millimetres. The results were assessed as presence/absence of BSH and as a semi-quantitative assessment (Table 4). Positive zones of precipitation indicated BSH activity. The assay aimed to semi-quantify BSH and hypothesised that larger zone sizes equated to increased BSH activity and smaller zones to less BSH activity. Other studies have reported varying results for BSH quantification using the agar plate assay (Begley *et al*., 2005a). Results were graphed and variations in BSH activity were analysed between the different isolates and between food/environmental and clinical groups (Figure 31 and Figure 32). Results were also compared with the corresponding bile resistance profiles to assess if the findings equated. For results to compare, a higher bile resistance corresponded with increased BSH activity and a lower bile resistance corresponded with decreased BSH activity. A positive and negative control was tested with each batch of GDCA/TDCA agar plates. *L. monocytogenes* EGDe was used as the BSH positive control and *L. innocua* was used as the BSH negative control. *L. monocytogenes* EGDe demonstrated definitive zones of BSH activity after 48 and 72 hours for both GDCA and TDCA, with the TDCA zone sizes smaller than GDCA zones. *L. innocua* was negative for BSH activity at both time points.

There were more reactions observed with the bile acid GDCA than TDCA. The majority of isolates recorded positive precipitation zones and greater zone size

variation using GDCA MRS agar. 72-hour zone sizes were larger than the zones read at 48 hours for both GDCA and TDCA. There was only one food/environmental culture negative for BSH activity at both time points: seafood isolate 1388. This isolate also had the lowest bile resistance profile. Overall, the clinical isolates resulted in fewer GDCA positive BSH zones than the food/environmental group. Four clinical strains, G-036, G-040, MQ130033 and MQ150011, had no reaction with GDCA at either time points. Isolate F347/17, an environmental isolate, produced the largest BSH zone of all the isolates after 48 hours at 10 mm while G-011 recorded the largest zone at 9 mm for the clinical isolates. After 72 hours the zone sizes were much larger for several isolates and there was less variation. Isolate 1203, an environmental (floor) isolate recorded the largest zone size of 12 mm and G-007, G-008, G-009, G-011 recorded zones of 10 mm, the largest of the clinical strains. The average GDCA zone size for the food/environmental isolates after 48 hours was 6.20 mm SD +/- 1.40 and 5.74 mm SD +/- 1.91 for the clinical isolates. The average zone size for the GDCA food/environmental isolates after 72 hours was 7.32 mm SD +/- 1.77 and 6.71 mm SD +/- 2.09 for the clinical isolates. There was little difference between the two groups zone sizes.

In comparison, the TDCA plates did not work as well and BSH activity was difficult to assess. As a result, there were fewer positive results using TDCA. A large number of the isolates recorded no active BSH activity and it was difficult to determine if the results were an accurate reflection of the true result or if the assay required additional optimisation for TDCA. When plates were read at 48 hours, 149/228 isolates had no detectable BSH activity and after 72 hours, 114 isolates still had no detectable BSH levels. There were more reactions with the food/environmental isolates than with the clinical and zone sizes were much smaller than with GDCA. The average TDCA zone size for the food/environmental isolates after 48 hours was 1.48 mm SD +/- 1.83 and 0.47 mm SD +/- 0.95 for the clinical isolates. The average TDCA zone size for the food/environmental isolates after 72 hours was 1.81 mm SD +/- 2.06 and 1.46 mm SD +/- 1.71 for the clinical isolates, much lower values than reported

for GDCA. The lack of detectable BSH activity and the small zone sizes made it difficult to utilise the TDCA results to quantify BSH levels in the isolates sufficiently.

Isolates of interest include the two mother and infant pairs represented in the clinical isolates. Results were comparable between the mother and infant for both GDCA and TDCA at both time points; MQ140011 and MQ140012 GDCA 5 mm and 6 mm respectively at both 48 and 72 hours, TDCA no reaction; MQ140034 and MQ140035 GDCA 5 mm and 6 mm respectively, TDCA no reaction. The average zone sizes for the food/environmental isolates were slightly larger than the clinical isolates for both GDCA and TDCA at 48/72 hour but the difference was minimal. Increased levels of BSH, potentially, signifies the ability of *L. monocytogenes* to detoxify bile more efficiently. Results indicate that the food and environmental strains have the ability to produce increased levels of BSH when compared to the clinical isolates, however, the differences between the two groups are slight, as displayed in figure 31 and 32.

An unpaired t-test was performed on the GDCA results to compare the food/environmental and clinical groups. It should be noted that a number of isolates did not have a BSH reaction with TDCA so statistical analysis was not applied. There was a statistical difference between the means of the two data sets at both time points for GDCA: p value = 0.03 at 48-hours and 72-hour p value = 0.007, indicating that the food/environmental isolates had greater BSH activity when compared to the clinical isolates.

Results by Lineage

GDCA and TDCA bile plate results were grouped by lineage to assess whether lineage influences the production of BSH. The majority of the isolates had lineage information available (215/228). Isolates were grouped into lineage I or lineage II. 99 isolates were identified as lineage I of which 61 were food or environmental isolates and 38 were clinical. 116 isolates were identified as lineage II of which 88 were food or environmental isolates and 28 were clinical. The means of the samples from Lineage I and Lineage II were compared for GDCA. GDCA 48-hour results were not significantly different with a p value = 0.12. Lineage II isolates had statistically larger

GDCA zone sizes than Lineage I at 72 hours with p values p = < 0.001. Statistical analysis was not applied to the TDCA results as a number of the isolates did not have a BSH reaction with TDCA. The bile acid GDCA was deemed a better predictor for BSH activity in this study. Results indicate that lineage, potentially, could have an effect on the production of BSH, however, additional isolates for both lineages are required for a more detailed analysis.

Results by Clonal Complex

The 48-hour BSH results were used to compare isolates by clonal complex to assess if individual clonal groups have the ability to produce more or less BSH (Figure 33, Figure 34). CC information was available for 210/228 of the isolates. CC121 represented the largest group with 43 isolates comprising of 38 food/environmental and 5 clinical isolates. A variety of different CC were represented including CC1, CC2, CC4, CC6, commonly isolated from clinical cases, and CC9 and CC121 commonly associated with food and environmental isolates.

Overall, the BSH results varied between the different CCs and isolates from within the same CC. It was difficult to identify any pattern of BSH activity. Isolate 1388 was the only food/environmental isolate not to have any BSH reaction with GDCA or TDCA. 1388 was grouped into CC2. Other CC2 isolates had larger BSH zone sizes with 1375, a meat isolate, recording a GDCA zone size of 9 mm, the isolate had no reaction with TDCA. 2237, an environmental isolate recorded the second smallest CC2 GDCA zone at 4 mm and had no reaction with TDCA. The mean GDCA zone size for CC2 was recorded at 5.90 mm SD +/- 1.97.

Interestingly, 8/18 of the CC2 isolates and 23/43 of the CC121 isolates recorded TDCA reactions, with a mean of 1.35 SD +/- 1.56 and 1.95 mm SD +/- 1.98 respectively. Other CC groups had no reaction with TDCA. Isolates from CC3 (4 isolates), CC8 (6 isolates), CC20 (5 isolates), CC87 (2 isolates), CC90 (2 isolates), CC224 (1 isolate), CC226 (1 isolate) and CC412 (1 isolate), had no reaction with TDCA (Figure 34).

CC1 and CC18 isolates had relatively high bile resistance profiles when assessed with the bile tolerance assay. In comparison with the BSH agar plate results, CC1 demonstrated increased BSH activity averaging at approximately 7.09 mm SD +/-

1.10 for GDCA and 1.23 mm SD +/- 1.96 for TDCA. CC18 isolates had a mean value of 6.46 mm SD +/- 1.33 for GDCA and 1.71 mm SD +/- 2.19 for TDCA. The BSH results for CC1 and CC18, for the majority, correlate with the bile resistance results indicating that these CCs are potentially more bile tolerant and have the ability to produce BSH at a good level. CC121 the largest CC group, had variations in BSH activity between isolates in the group with an average GDCA zone size of approximately 7.05 mm SD +/- 1.01 and 1.95 mm SD +/- 1.98 for TDCA. 20/43 CC121 isolates had no reaction with TDCA and isolates that did react, resulted in varied zone sizes. CC9, another food related CC, had a lower BSH mean than CC121 at 5.39 mm SD +/- 1.21 for GDCA and 0.79 mm SD +/- 1.41 for TDCA. In comparison, CC9 and CC121 had similar bile resistance profiles with means of 84%. Overall, the CC results varied for BSH activity. Larger CC group sizes with comparable numbers of food/environmental and clinical isolates would be of benefit to further investigate the influence of CC on BSH activity.

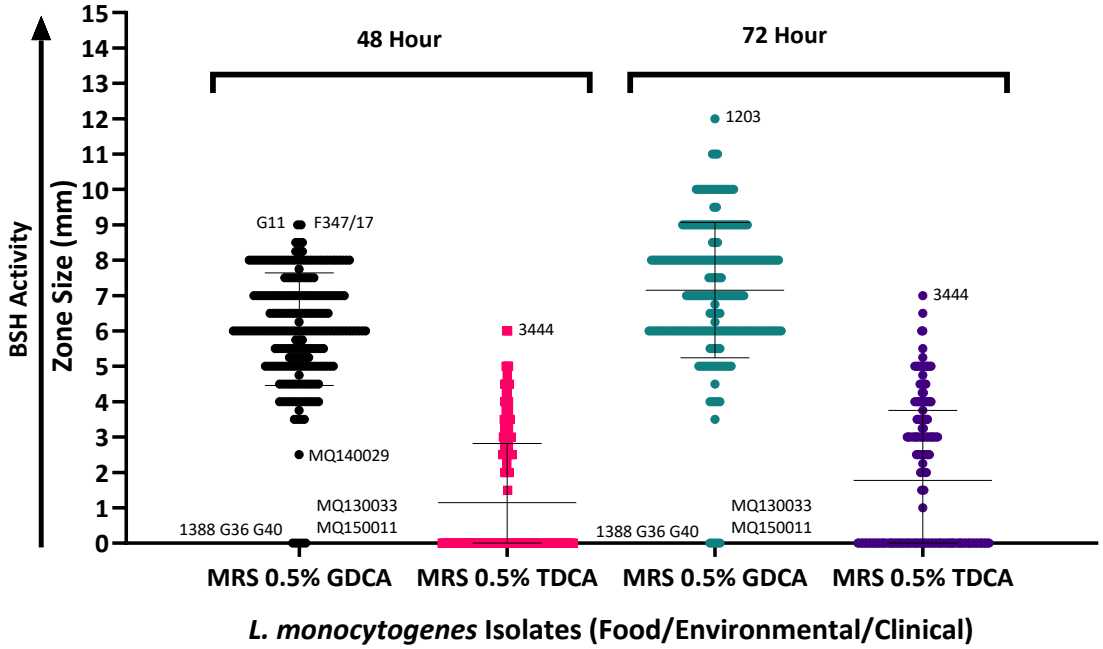


Figure 31. BSH levels in **food, environmental and clinical isolates**. Cultures were patch inoculated onto MRS agar supplemented with 0.5% (wt/vol) GDCA/TDCA and incubated anaerobically. BSH zone size diameters (mm) were measured at 48- and 72- hours. It was hypothesised that larger zone sizes indicated increased BSH activity and smaller zones, less BSH activity. The GDCA MRS plates produced more positive BSH results than TDCA at both 48 and 72 hours, zone sizes were also larger. Samples were tested in biological and technical duplicate. The error bars represent the mean +/- standard deviation.

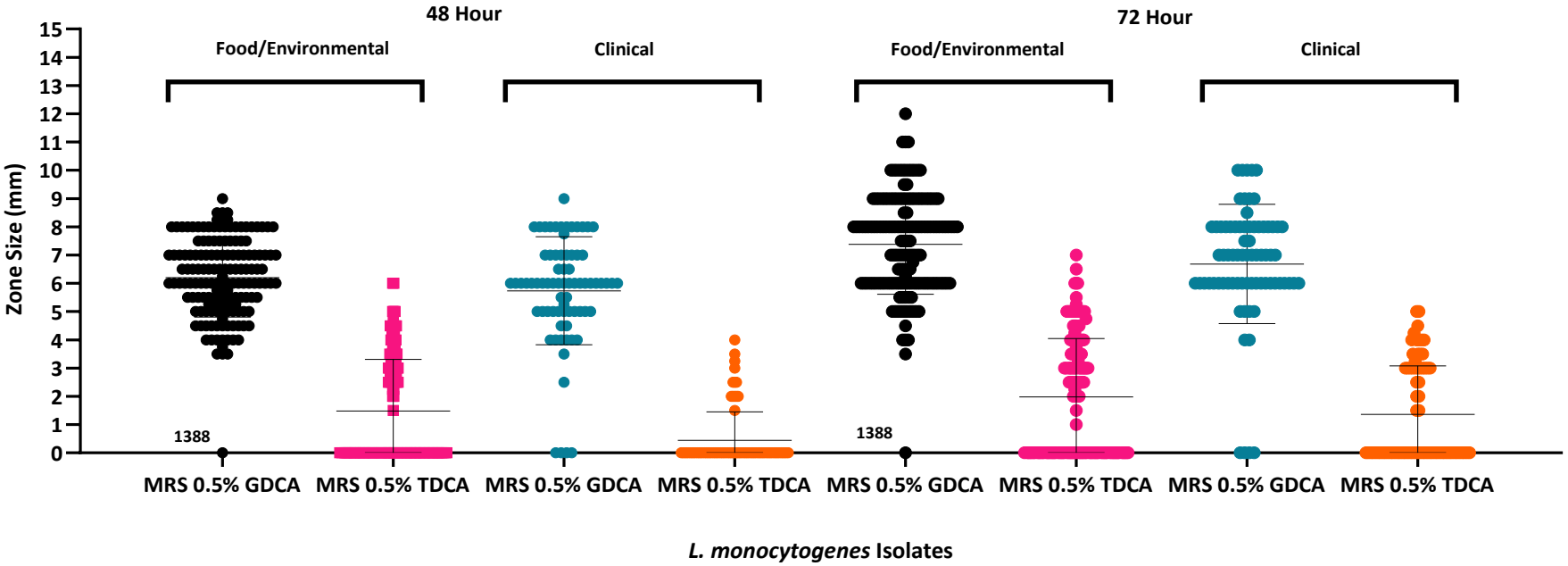


Figure 32. BSH levels in **food/environmental isolates versus clinical isolates**. Cultures were patch inoculated onto MRS agar supplemented with 0.5% (wt/vol) GDCA/TDCA and incubated anaerobically. BSH zone sizes (mm) were measured after 48- and 72-hours and plotted by sample origin (food/environmental or clinical). It was hypothesised that larger zone sizes indicated increased BSH activity. The GDCA plates produced more positive BSH results than TDCA. The food/environmental isolates recorded slightly higher levels of BSH activity for both GDCA and TDCA compared to the clinical isolates. Samples were tested in biological and technical duplicate. The error bars represent the mean +/- standard deviation.

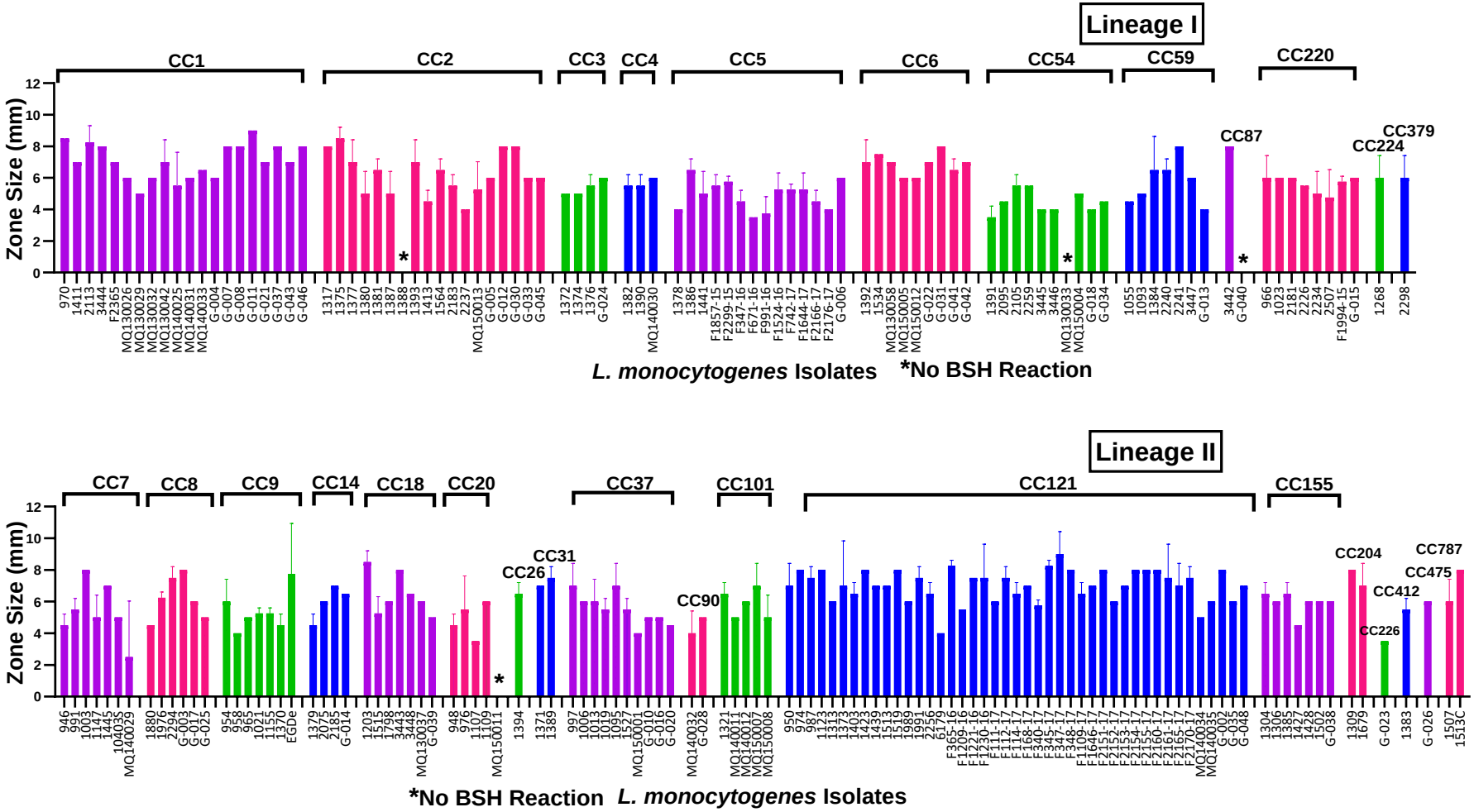

Lineage I
CC1
CC2
CC3
CC4
CC5
CC6
CC54
CC59
CC220
CC87
CC224
CC379
Zone Size (mm)
0
2
4
6
8
10
12
970
1411
2113
3444
F2365
MQ130026
MQ130029
MQ130032
MQ130042
MQ140025
MQ140031
MQ140033
G-004
G-007
G-008
G-011
G-021
G-037
G-043
G-046
1317
1375
1377
1380
1381
1387
1388
1393
1413
1564
2183
2237
MQ150013
G-005
G-012
G-030
G-033
G-045
1372
1374
1376
G-024
1382
1390
MQ140030
1378
1386
1441
F1857-15
F2299-15
F347-16
F671-16
F991-16
F1524-16
F742-17
F1644-17
F2166-17
F2176-17
G-006
1392
1534
MQ130058
MQ150005
MQ150012
G-022
G-031
G-041
G-042
1391
2095
2105
2259
3445
3446
MQ130033
MQ150004
G-018
G-034
1055
1093
1384
2240
2241
3447
G-013
3442
G-040
966
1023
1181
2226
2234
2507
F1994-15
G-015
1268
2298
L. monocytogenes Isolates
*No BSH Reaction
Lineage II
CC7
CC8
CC9
CC14
CC18
CC20
CC37
CC101
CC121
CC155
CC204
CC787
CC475
CC412
CC226
Zone Size (mm)
0
2
4
6
8
10
12
946
991
1003
1147
1445
10403S
MQ140029
1880
1976
2294
G-003
G-025
954
958
965
1021
1155
1370
EGDe
1379
2075
2185
G-014
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3443
3448
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G-039
948
976
1107
1109
MQ150011
1394
1371
1389
CC31
CC26
997
1006
1013
1019
1095
1527
MQ150001
G-010
G-016
G-020
MQ140032
G-028
CC90
1321
MQ140011
MQ140012
MQ150007
MQ150008
950
974
987
1123
1313
1333
1493
1439
1513
1519
1989
1991
2256
6179
F365-16
F1209-16
F1221-16
F1230-16
F111-17
F112-17
F114-17
F168-17
F340-17
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F348-17
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F2151-17
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F2155-17
F2160-17
F2161-17
F2176-17
MQ140034
MQ140035
G-002
G-035
G-048
1304
1306
1385
1427
1428
1502
G-038
1309
1679
G-023
1383
G-026
1507
1513C
*No BSH Reaction
L. monocytogenes Isolates

Figure 33. 48-hour **0.5% GDCA MRS agar** BSH zone size results were grouped by **clonal complex** group to identify potential patterns relating to BSH activity. It was hypothesised that larger zone sizes indicated increased BSH activity. BSH activity varied between the different CCs and amongst individual CC groups. Four isolates (with available CC information) had no detectable BSH activity: 1388 (CC2), MQ150011 (CC20), MQ130033 (CC54) and G-040 (CC87). Samples were tested in biological and technical duplicate. The error bars represent the mean +/- standard deviation.

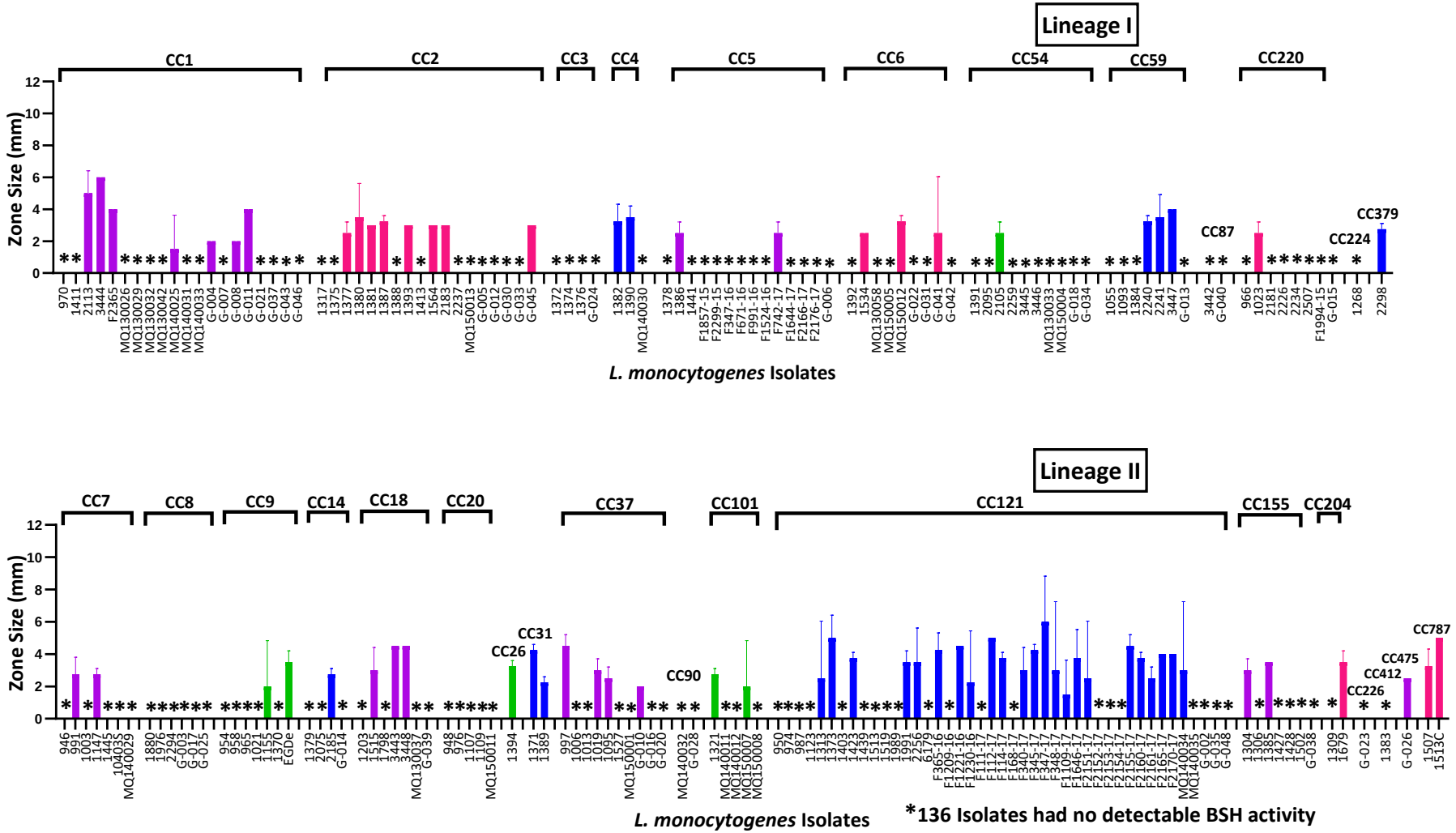

Lineage I
CC1
CC2
CC3
CC4
CC5
CC6
CC54
CC59
CC220
Zone Size (mm)
CC87
CC224
CC379
L. monocytogenes Isolates
Lineage II
CC7
CC8
CC9
CC14
CC18
CC20
CC37
CC101
CC121
CC155
CC204
CC26
CC31
CC90
CC226
CC412
CC475
CC787
L. monocytogenes Isolates
*136 Isolates had no detectable BSH activity

Figure 34. 48-hour **0.5% TDCA MRS agar** BSH zone size results were grouped by **clonal complex** group to identify potential patterns relating to BSH activity. It was hypothesised that larger zone sizes indicated increased BSH activity. BSH activity varied between the different CCs and amongst individual CC groups. The error bars represent the standard deviations of the duplicate replicates. BSH activity was not detected for 136/210 of the isolates. TDCA produced very few positive BSH zones in comparison with GDCA and zone sizes were much smaller. Samples were tested in biological and technical duplicate. The error bars represent the mean +/- standard deviation.

Bile Salt Hydrolase-Bioluminescence Probe Assay

The enzymatic probe assay was originally developed to quantify BSH levels in Lactobacillus cultures. A small selection of the *L. monocytogenes* cultures were trialled with the assay conditions developed for Lactobacillus cultures. Bile resistance results for the cultures grown anaerobically were reviewed and a "higher" and a "lower" bile resistance isolate was selected from the food/environmental group and from the clinical group. Two food/environmental cultures, 944 (higher bile resistance) and 948 (lower bile resistance) and two clinical cultures, G-003 (lower bile resistance) and G-039 (higher bile resistance) were tested. *L. monocytogenes* EGDe was used as the BSH positive control and *L. innocua* as the BSH negative control. The principle of the assay is based on BSH activity freeing caged luciferin and "free" luciferin producing light when it reacts with luciferase (Figure 12). The level of luminescence emitted equates to levels of BSH in the sample (Figure 35). Two different bile acids were used, cholic acid and deoxycholic acid. Samples were mixed with enzymatic detection probes and aliquoted into 96 well plates coated with 4T1-RedLuc cells, a mouse cell line transformed with luciferase to detect luciferin. Control samples were prepared by mixing cell free extracts with aminoluciferin in mercaptoethanol. Luminescence, indicating BSH activity, was measured using an IVIS imaging system and associated software. Only one of the samples tested, G-003, produced a detectable signal for both bile acids, at levels of 7.09E+05 for cholic acid and 7.81+05 pp/sec/cm^2/sr for deoxycholic acid. Samples 944, 948, G-039 and control EGDe emitted limited signal and bile acid levels could not be quantified (Figure 36). Further work is required to optimise the assay for *L. monocytogenes* cultures.

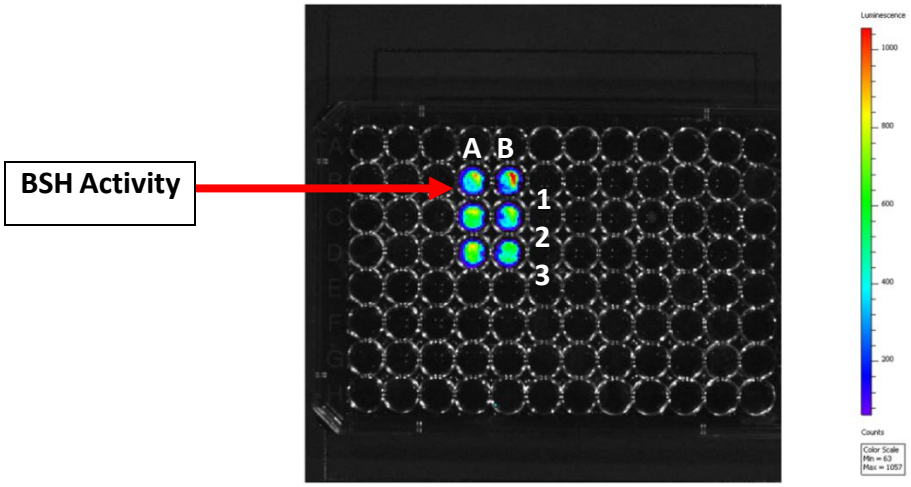


Figure 35. BSH Probe Assay Plate. Samples were mixed with enzymatic detection probes (cholic acid, deoxycholic acid) and aliquoted into 96 well plates coated with 4T1-RedLuc cells. The mouse cell line is transformed with luciferase to detect luciferin. BSH activity frees caged luciferin which reacts with luciferase to emit light. An IVIS imaging system was used to measure luminescence, indicating BSH activity. Only one sample in the trial, G-003, emitted a detectable signal (**A 1-3** Sample G-003 Cholic Acid, **B 1-3** Sample G-003 Deoxycholic Acid).



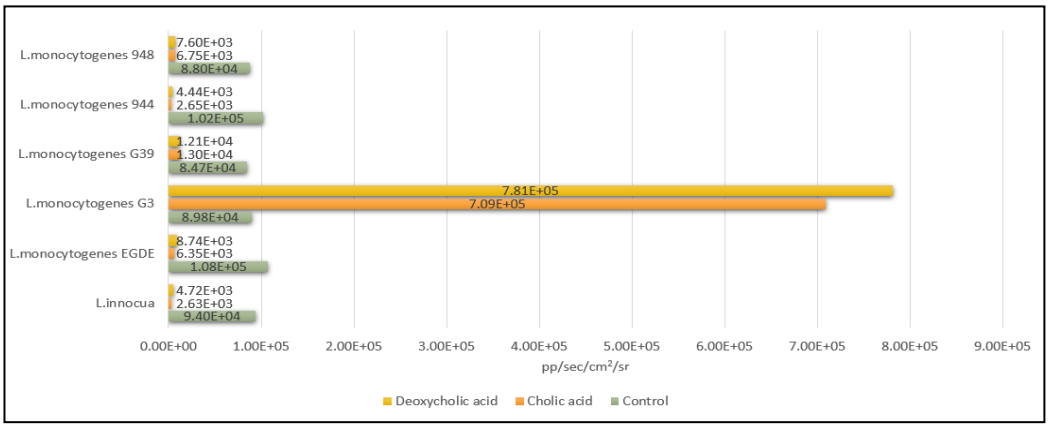


Figure 36. BSH Enzymatic Bioluminescence Levels. Luminescence, indicating BSH activity, was measured using an IVIS imaging system and associated software. Emitted bioluminescence levels were measured for deoxycholic acid, cholic acid and control samples. Only one clinical sample G-003 resulted in detectable levels of BSH: cholic acid 7.09E+05, deoxycholic acid 7.81+05 pp/sec/cm^2/sr. Samples were tested in biological triplicate.

Bile Salt Hydrolase Polymerase Chain Reaction-Detection

A selection of isolates were screened for the *bsh* gene using PCR and gel electrophoresis. Results from the bile resistance assay were reviewed and a selection of "higher" and "lower" isolates (anaerobic growth), including food/environmental and clinical isolates, were selected to analyse. Results demonstrated that all the isolates screened were positive for the *bsh* gene as visualised by an 87 base pair band on the agarose gel (Figure 37). *L. monocytogenes* EGDe was used as the *bsh* positive control and *L. innocua* as the *bsh* negative control.

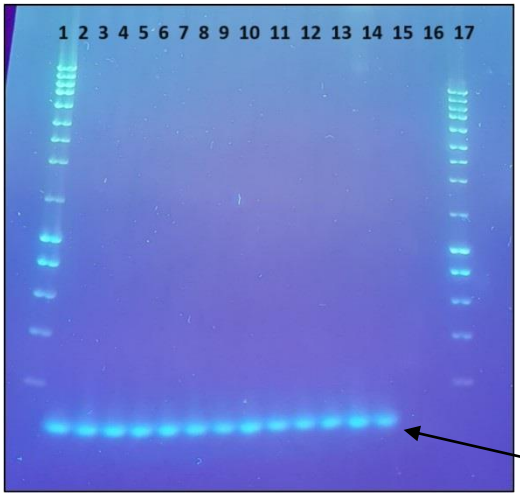


Figure 37. *bsh* Gel Electrophoresis. Samples were analysed for the presence of the *bsh* gene by PCR. PCR products were run on a 1.5% agarose gel stained with SYBR Safe at 100V. A UV transilluminator was used to detect an 87 base pair band for *bsh*. All *L. monocytogenes* isolates tested were positive for the *bsh* gene. *L. innocua*, the negative control was *bsh* negative. Lane 1 Ladder III Marker, Lane 2 944, Lane 3 948, Lane 4 950, Lane 6 987, Lane 7 1101, Lane 8 MQ140030, Lane 9 MQ140032, Lane 10 MQ140033, Lane 11 MQ150011, Lane 12 MQ150012, Lane 13 MQ150013, Lane 14 *L. monocytogenes* EGDe (*bsh* positive control), Lane 15 *L. innocua* (*bsh* negative control), Lane 16 No Template Control, Lane 17 Ladder III.

Bile Salt Hydrolase Quantitative PCR

A small number of isolates were selected to screen for *bsh* gene expression by reverse transcription and quantitative real-time PCR. The development of the PCR aimed to compliment the traditional agar plate assay for bile salt hydrolase quantification. Bile resistance results for the cultures grown anaerobically were reviewed and a "lower" bile resistance isolate was selected from the food/environmental group (948) and a "higher" (G-039) and a "lower" (G-003) bile resistance isolate selected from the clinical group. *L. monocytogenes* EGDe was used as the positive control. Samples were tested after 6- and 24-hour incubation in pH 7.5 BHI broth to determine levels of *bsh*. Two time points were selected to assess the effect of growth phase on *bsh* gene expression. 6-hour represented the exponential phase of growth and 24-hour represented the stationary phase of growth. RNA was extracted from samples and reverse transcribed into cDNA. Real-Time PCR was used to quantify *bsh* gene expression. Gene expression was calculated by the ratio between the sample *bsh* gene concentration and the housekeeping gene. The 6-hour samples resulted in lower levels of *bsh* gene expression in comparison with the 24-hour samples. Positive control EGDe had the highest level of *bsh* out of the four samples tested after 6 hours of incubation. Levels slightly increased after 24 hours for control EGDe. The 24-hour results indicated that the clinical samples (G-003 and G-039) had the highest levels of *bsh* expression (Figure 38). Future experiments should include *L. innocua* (*bsh* negative) as the negative control. Further work is required to develop and optimise the assay for all isolates.

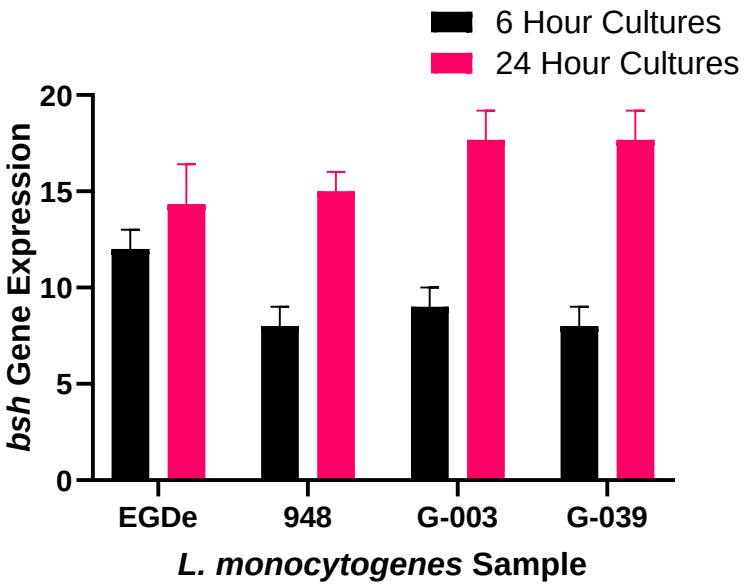


Figure 38. *bsh* Quantitative PCR. A selection of samples were screened for *bsh* gene expression. 6-hour and 24-hour cultures were trialled to represent the exponential and stationary phases of growth. RNA was extracted from samples and reverse transcribed into cDNA. Real-time PCR was used to quantify *bsh* levels in samples. Gene expression was calculated by the ratio between the sample *bsh* gene concentration and the housekeeping gene. The 24-hour cultures resulted in higher levels of *bsh* gene expression than the 6-hour cultures. Samples were tested in biological triplicate. The error bars represent the mean +/- standard deviation. Analysis was completed with Bríona O'Mara. Further work is required to develop and optimise the assay.

Discussion

In this study the effect of bile and anaerobic stress on *L. monocytogenes* was assessed using 228 isolates from food, environmental and clinical sources. The food and environmental group comprised of 154 isolates and the clinical group represented 74 isolates. Whole Genome Sequencing information was available for 215 out of 228 of the strains: 149 food/environmental and 66 of the clinical strains. WGS information was used to identify lineage and clonal complex. 25 of the clinical strains had previously been sequenced and sequence information made available (O'Callaghan *et al*., 2017).

The study aimed to assess the effect of bile and anaerobic stress on *L. monocytogenes* isolates and to identify potential differences in resistance patterns between clinical and food/environmental sources. *L. monocytogenes* is exposed to various stressors in the human gastrointestinal tract, including stomach acid, bile, a lowered pH and a lowered oxygen availability. The bacteria must be able to overcome these hurdles to establish infection. The gastrointestinal phase of *L. monocytogenes* infection is not as well studied compared to our current knowledge on cellular invasion and bloodstream infection. It was hypothesised that the clinical strains may be more bile tolerant. Experiments were performed under conditions representative of those in the gut including a lowered pH and anaerobic conditions. Results were further analysed by lineage and clonal complex to identify any links related to bile resistance.

The effect of bile on *L. monocytogenes* isolates was assessed using two methods, a bile broth assay for resistance and an agar plate assay to detect BSH activity. Development work was required to optimise the assays for the large number of samples. Trial experiments for BSH activity were also carried out using an enzymatic probe assay and a quantitative PCR approach. Strain variation was observed for both bile resistance and BSH activity. Similar studies have also reported that bile resistance may be strain specific and cannot be generalised by species (Begley *et al*., 2005a, Olier *et al*., 2004, Wright *et al*., 2016).

Isolates grown aerobically exhibited a high level of bile tolerance in comparison to the same cultures grown anaerobically. Food, environmental and clinical isolates, grown aerobically, had similar bile resistance patterns and consistently high levels of tolerance. There was little variation across the isolates or between the food/environmental and clinical groups. In contrast, the cultures grown anaerobically had much more varied bile resistance patterns and were less tolerant of bile. The effect of anaerobic growth is not as well understood as aerobic growth and the data generated in this study provides an insight into the impact of environmental conditions on bile resistance. Results do not support the theory that anaerobic conditions may trigger resistance mechanisms in *L. monocytogenes* to detoxify bile. However, the assay developed in this study only exposed the cultures to bile for a short time period. Other studies used longer periods of exposure, allowing the bacteria to adapt to the environment (Begley *et al*. 2002, Begley *et al*. 2005a, Dowd *et al*., 2011). The one-hour bile exposure provided a "snap-shot" into the bile tolerance profiles of the various isolates and we propose that this models the early phase of bile exposure in the GI tract. The assay system was effectively used as a comparative tool to characterise individual strains, different groups (food, environmental, clinical) and genetic determinants such as lineage and CC.

The anaerobic cultures had slower growth rates compared to cultures grown aerobically. This was factored into the assay to ensure comparable growth. However, the aerobic cultures may have entered the stationary phase of growth before the anaerobic cultures. Studies have shown that growth phase can affect bile tolerance with exponential phase cultures more affected by bile than stationary phase cultures. Begley *et al*., 2002 demonstrated that stationary phase cultures of *L. monocytogenes* LO28, grown anaerobically, tolerated concentrations of porcine, bovine, human bile and bile acids, greater than those found in vivo. In comparison exponential phase LO28 cultures were more sensitive to unconjugated bile acids. pH also has an effect on bile tolerance. Studies have also shown that reducing the pH of gallbladder bile to pH 5.5 causes an increase in toxicity and specific resistance mechanisms, including the general stress-response regulator Sigma factor B (*sigB*) (Begley *et al*., 2005a,

Dowd *et al*., 2011), *bsh* (Begley *et al*., 2005a, Dussurget *et al*., 2002) and the bile exclusion system (*bilE*) (Sleator *et al*., 2005), are required for survival. Trial work, performed as part of this thesis, confirmed that cultures exposed to pH 5.5 were less tolerant of bile than at pH 7.5. This has implications for mimicking gut infection where the pH of the duodenum (the site of the organisms first exposure to high concentrations of bile acids) is likely to be mildly acidified by mixing with chyme/stomach acids (Campbell., 2009, Dowd *et al*., 2011).

The agar plate assay proved relatively difficult to perform and to assess BSH activity accurately. There was more BSH activity observed for the bile acid GDCA when compared to TDCA. As a result, we found that GDCA was a better predictor of BSH activity than TDCA. Human bile has a higher ratio of glycoconjugates to tauroconjugates at approximately 3 to 1 (Begley *et al*., 2005b). This is in contrast with murine bile acid composition which contains a predominance of tauro-conjugated bile acids (Begley *et al.,* 2005b). Studies have shown that glycoconjugated bile acids demonstrate increased antibacterial effects at low pH and may be responsible for the inhibitory effects when the pH of gallbladder bile is decreased (Begley *et al*., 2005a, Dowd *et al*., 2011). The agar plate cultures were grown under standard environmental conditions prior to inoculation onto the bile plates. Begley *et al*., 2002 reported that adapting cultures prior to bile exposure increased resistance. Cultivating isolates anaerobically for the BSH assay prior to inoculation onto bile plates, may have induced BSH activity more effectively. Müller-Herbst *et al*., 2014 deduced that anaerobic conditions could be an environmental signal to induce virulence gene expression.

To our knowledge no study has correlated zone size from BSH activity agar plates with actual potency of BSH or amount of BSH protein present. An alternative method to quantify *L. monocytogenes* functional BSH activity may be more accurate than the agar plate assay. A quantitative bioluminescence-based approach for BSH activity was recently developed by Khodakivskyi *et al.,* 2021. In addition, the development of a quantitative PCR for *bsh* gene expression was examined as part of this study. The bioluminescence BSH probe assay and the quantitative PCR based method were

trialled herein using a small selection of samples. The bile resistance results were reviewed and a selection of higher and lower bile tolerant strains from the food/environmental and clinical groups were assessed with the assays. Four isolates (two food/environmental, two clinical) were trialled with the BSH enzymatic assay but only one clinical sample produced a detectable signal. Three strains (one food/environmental, two clinical) were tested with the molecular assay to quantify *bsh* gene expression with varying levels detected. Both methods have the potential to quantify BSH more effectively than the agar plate assay. However, further work is required to optimise the assays for *L. monocytogenes* cultures and to equate BSH activities with other accurate measures of activity such as chemical assessment (UPLC-MS assessment) of rates of bile acid conversion in culture.

Interestingly, the bile resistance results for the anaerobically grown isolates identified the clinical strains as significantly more bile tolerant than the food/environmental strains ($p = < 0.001$). This is a significant finding and demonstrates the evolutionary pressure on these isolates to survive in the gut. We may be witnessing loss of virulence for the food strains. There is a potential for *L. monocytogenes* to lose virulence factors (Maury *et al.,* 2017, Nightingale *et al*., 2005, Jacquet *et al*., 2004). An example is Internalin A which is a key virulence factor for *L. monocytogenes*. Loss of internalin A function may alter the phenotype (Jacquet *et al*., 2004, Nightingale *et al*., 2005, Van Stelten *et al*., 2016). Approximately 50% of *L. monocytogenes* food isolates have a mutation in the *inl*A gene. Differences in virulence between strains may exist (Hain *et al*., 2007) with epidemiology data implying that not all *L. monocytogenes* strains are equally virulent. In contrast, the food and environmental strains recorded increased BSH activity when compared with the clinical strains, indicating the ability to detoxify bile to a greater extent (GDCA $p = 0.03$ at 48 hours and 72-hour $p = 0.007$). Statistical analysis did not correlate results between bile resistance and BSH activity. For results to correlate, it was hypothesised that a higher bile resistance corresponded with increased BSH activity and a lower bile resistance with decreased BSH activity. Four clinical strains, G-036, G-040, MQ130033 and MQ150011, did not have BSH reactions with GDCA indicating a

decreased ability to detoxify bile. In comparison, the same isolates had higher bile resistance profiles (G-036 97%, G-040 88%, MQ130033 91%, MQ150011 91%) indicating that they are quite tolerant of bile. Similar findings were reported by Begley *et al*., 2005a where levels of BSH activity did not correlate with corresponding bile tolerance profiles for isolate LO28, EGDe and a selection of other *L. monocytogenes* strains. EGDe was tested in this study and the bile resistance result did correlate with BSH activity. However, the results for other isolates did not correlate between the two assays. There is a possibility, that the zones of precipitation observed on the bile agar plates may require additional factors to accurately predict BSH activity or that local acidification of the media (which varies between strains) may influence the size of the zone.

Seafood isolate 1388 was identified as one of the most interesting strains in our study. The isolate had the lowest bile resistance of all 228 isolates with a mean of 56% tolerance and it was the only food and environmental isolate that did not record BSH activity with either GDCA or TDCA. Results indicate that the isolate is not as tolerant of bile as other *L. monocytogenes* strains studied. To investigate, future work should include analysing isolate 1388 for the presence of the *bsh* gene using the PCR based method and gene expression by reverse transcription quantitative PCR. The same isolate was studied by a partner lab in a multi-disciplinary project where 1388 was also identified as an isolate of interest (Wu *et al*., 2022). The isolate was found to have reduced SigB activity due to mutations in the *sigB* operon. As a result, the isolate had increased tolerance to salt stress and decreased resistance to low pH. SigB is required for survival in the GI tract and for entry into epithelial cells (Garner *et al*., 2006). Begley *et al*., 2005ab demonstrated that *sigB* mutations reduced the ability of *L. monocytogenes* LO28 to tolerate bile. Transcription of *bsh* is also affected. BSH has a vital function in bile tolerance and is regulated by SigmaB. A study by Dowd *et al*., 2011 revealed that specific resistance mechanisms including SigmaB are required for survival of *L. monocytogenes* when exposed to bile at a lower pH (pH5.5). Zhang *et al*., 2011 also demonstrated that *sigB* deletion resulted in reduced bile tolerance. Isolate 1388 was grouped into CC2, commonly associated with clinical

cases of listeriosis. Other CC2 isolates studied had higher resistance patterns and increased BSH activity.

There were two mother and infant isolate pairs represented by the clinical strains. The bile resistance profiles for the mother and infant were comparable and they reported very similar BSH activity: MQ140011 and MQ140012 anaerobic growth bile tolerance of 89% and 91% respectively, GDCA BSH zone 5 mm and 6 mm, TDCA no reaction; MQ140034 and MQ140035 anaerobic growth bile tolerance of 95% and 97% respectively, GDCA BSH zone 5 mm and 6 mm, TDCA no reaction. As these are likely to represent effectively the same strain, this provides confidence that the assay conditions developed in our study are highly accurate and predictive.

Clinically unrelated strains MQ150007 and MQ150008 differed by two SNPs and were thought likely to be phylogenetically linked (Hilliard *et al*., 2018). However, no epidemiological link was previously determined. The strains were isolated from separate patients within a one-month period. These strains are similar and in our study both isolates had equivalent bile resistance patterns and BSH activity levels except for the TDCA results; MQ150007 was positive for BSH and MQ15008 had no reaction. In comparison, the same closely related isolates were studied by a partner lab in a multi-disciplinary project for a different type of phenotypic stress, osmotic pressure (Wu *et al*., 2022). Both isolates had different phenotypes when exposed to osmotic stress. MQ150008 demonstrated reduced growth under osmotic stress in comparison to MQ150007. The combined results from both studies may suggest that these are very different strains that are genetically similar but phenotypically distinct and that genetic similarity may depend on other factors. Analysis of phenotype contributes to the understanding of the capacity of these isolates to cause illness.

Analysis of the genetic relationship between lineage/CC with bile tolerance results concluded that lineage has no apparent effect on bile resistance. In comparison, Wu *et al*., 2022, who studied the same isolates, identified lineage I as a predictor for a different phenotype, salt tolerance. BSH activity, as determined by the agar plate assay, concluded that there was no correlation with lineage for the GDCA 48 hours results but did identified Lineage II isolates as having increased GDCA BSH activity

after 72 hours. Analysis by CC, similar to lineage, demonstrated varied results. There was correlation between the bile resistance results and BSH activity for CC1, represented by 5 food/environmental and 15 clinical isolates and CC18 comprising of 5 food/environmental and 2 clinical isolates. CC1 and CC18 generated the most interesting results, demonstrating higher levels of bile tolerance and increased BSH activity compared to other CCs. CC18 was identified as a clonal group of interest in another study using similar food and clinical isolates. Myintzaw *et al*., 2023 reported that CC18 isolates were significantly ($p \leq 0.05$) faster growing at 4 °C than other CC groups. The study suggested that differences in growth rates in cold conditions was associated with CC. A study by Hingston *et al*., 2017 determined that *L. monocytogenes* tolerance to food-related stressors including salt, acid, cold and desiccation varies according to serotype and CC. *L. monocytogenes* strains were screened phenotypically and WGS was used to identify genetic elements relating to stress tolerant and sensitive phenotypes. The study identified CC as a better predictor than serotype of salt and acid tolerance but not cold and desiccation tolerance. Strains with full length *inlA* had increased tolerance to the cold ($P < 0.001$) in comparison with strains with a premature stop codon in the *inlA* gene. The presence of a plasmid in strains displayed increased acid tolerance ($P = 0.013$). The study deduced that minor genetic differences can have an effect on the *L. monocytogenes* stress response (Hingston *et al*., 2017).

CC1 isolates are commonly isolated from clinical cases and are generally classed as more virulent than other CCs (Maury *et al.*, 2016). CC1 is strongly associated with Central Nervous System and Materno-Neonatal infection. Other clinical CCs analysed in the study included CC2, CC4 and CC6. Results for these groups varied. CC121, primarily food related, represented the largest clonal group in this study with five of the strains isolated from clinical sources. There was variation in the bile resistance patterns and BSH activity for CC121 strains. The five clinical CC121 strains had a high level of bile resistance and average BSH activity. However, other CC121 food/environmental isolates had higher levels of bile resistance and BSH activity. CC9, another food related group, had varying bile resistance and BSH activity. Larger

CC group sizes with comparable numbers of food/environmental and clinical isolates would allow for a more detailed analysis of the effect of CC on the bile response.

In conclusion, the effect of bile and anaerobic stress on a large set of *L. monocytogenes* isolates was quite varied for all strains studied. Results indicate that the bile response may be strain-specific or stress response mechanisms may differ between strains. A bile resistance assay was developed as part of this study and proved to be very reproducible. The assay incorporated a lowered pH and anaerobic growth conditions to mimic conditions in the gut where bacteria are exposed to the inhibitory effects of bile. Interestingly, the clinical isolates were shown to have higher levels of bile tolerance in comparison to food and environmental isolates. This is an important finding that may indicate the evolutionary pressure on these strains and could possibly be a predictor of hypervirulent strains. Anaerobic growth conditions were shown to increase the deleterious effect of bile on the strains. The study also investigated the link between bile tolerance and BSH activity. To our knowledge, this link has not been investigated on such a large scale to date. Although there was little evidence of a correlation between bile tolerance and BSH activity, other factors, not included as part of this work, may be involved, such as membrane integrity of the strains or the effect of efflux pumps such as MdrT or MdrL. Further work would be of benefit to establish if any other factor(s) has an effect on the relationship between bile tolerance and BSH. The results generated from this study provide an insight into the less studied gastrointestinal phase of infection and potential associations between bile tolerance of individual strains/lineages and efficacy of *L. monocytogenes* infection.

Overall Conclusions and Suggestions for Future Work

The study of a large set of *L. monocytogenes* isolates (228) from food, environmental and clinical sources revealed bile resistance variations between the different strains. Clinical isolates had increased bile resistance when compared to food and environmental isolates. Growth environment had a demonstrated effect with anaerobic isolates less bile tolerant than aerobic over the time period tested. Bile salt hydrolase activity was difficult to predict using the agar plate assay and an alternative method might be a better approach. Two different methods were trialled for BSH activity including an enzymatic probe assay and a quantitative PCR based method. Future work could include the development and optimisation of these assays to analyse functional BSH levels in the bank of isolates. Further work to investigate any potential links between lineage/clonal complex and bile resistance would benefit from additional isolates to represent the different clonal complexes

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Appendix

Bile Resistance Assay: Anaerobic Results

Table 2. **Anaerobic** Growth Bile Resistance Results. Difference between Time 0 and Time 1 values were used to calculate bile resistance. Samples were tested in biological and technical triplicates.

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
	Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3
944	93	95	96	**1389**	80	79	82	**F2365**	99	98	101	**MQ140034**	95	98	93
946	91	93	90	**1390**	84	86	86	**F1857-15**	74	75	72	**MQ140035**	93	100	98
948	86	85	83	**1391**	84	80	83	**F1994-15**	84	83	84	**MQ150001**	94	96	95
950	93	95	92	**1392**	98	96	95	**F2242-15**	80	79	79	**MQ150004**	94	97	97
954	89	92	90	**1393**	82	86	86	**F2246-15**	80	80	81	**MQ150005**	94	97	95
958	77	78	76	**1394**	78	78	76	**F2299-15**	76	76	75	**MQ150007**	91	91	94
960	90	88	88	**1403**	93	94	93	**F347-16**	91	92	91	**MQ150008**	91	90	88
965	82	84	79	**1411**	81	83	79	**F365-16**	79	79	79	**MQ150011**	91	91	92

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
	Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3
966	87	88	88	**1413**	80	77	76	**F671-16**	70	67	73	**MQ150012**	74	74	73
970	89	90	88	**1423**	80	77	76	**F991-16**	79	81	79	**MQ150013**	88	91	89
974	74	71	71	**1427**	82	83	79	**F1209-16**	83	85	87	**G-001**	95	93	93
976	92	89	88	**1428**	94	97	93	**F1221-16**	93	92	91	**G-002**	96	97	94
987	75	79	77	**1439**	81	81	76	**F1230-16**	87	84	86	**G-003**	88	85	85
991	92	91	90	**1441**	88	89	87	**F1524-16**	77	80	80	**G-004**	91	94	93
997	100	98	98	**1445**	73	73	73	**F111-17**	95	94	97	**G-005**	90	87	89
1003	75	76	73	**1502**	79	80	79	**F112-17**	86	89	90	**G-006**	89	89	92
1006	89	87	88	**1507**	84	84	88	**F113-17**	81	80	80	**G-007**	87	89	89
1013	93	92	95	**1513**	74	72	74	**F114-17**	73	76	76	**G-008**	89	89	89
1019	83	82	79	**1515**	101	99	102	**F168-17**	77	77	77	**G-009**	92	92	94
1021	92	90	91	**1519**	76	74	71	**F340-17**	82	81	79	**G-010**	92	93	91
1023	80	80	80	**1527**	92	94	91	**F345-17**	78	79	79	**G-011**	91	91	89
1055	81	81	81	**1534**	84	85	84	**F347-17**	81	79	81	**G-012**	87	90	90
1093	79	77	80	**1564**	81	85	81	**F348-17**	79	77	77	**G-013**	94	93	90
1095	79	79	80	**1673**	81	85	84	**F742-17**	78	78	79	**G-014**	95	98	95

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
	Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3
1101	95	96	95	**1679**	95	96	96	**F1099-17**	91	95	92	**G-015**	81	77	77
1107	78	78	80	**1734**	80	77	78	**F1109-17**	78	83	83	**G-016**	93	94	92
1109	56	56	60	**1798**	98	100	101	**F1644-17**	75	84	81	**G-017**	90	88	89
1123	77	76	77	**1880**	63	66	64	**F1646-17**	90	91	89	**G-018**	78	80	79
1147	92	95	94	**1976**	90	88	91	**F2151-17**	79	80	79	**G-019**	91	96	95
1155	95	91	93	**1989**	78	79	77	**F2152-17**	81	82	80	**G-020**	92	93	94
1203	97	97	98	**1991**	94	96	96	**F2153-17**	77	76	77	**G-021**	92	90	89
1268	84	85	88	**2075**	79	78	77	**F2154-17**	79	80	81	**G-022**	94	95	94
1304	99	94	98	**2095**	91	93	95	**F2155-17**	82	81	80	**G-023**	88	90	92
1306	99	96	97	**2105**	89	88	85	**F2160-17**	79	80	81	**G-024**	85	84	83
1309	79	78	78	**2113**	88	88	87	**F2161-17**	96	84	83	**G-025**	90	92	92

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
	Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3
1313	88	91	91	**2181**	89	91	90	**F2165-17**	79	78	79	**G-026**	101	100	99
1317	61	61	61	**2183**	83	83	83	**F2166-17**	85	85	85	**G-027**	100	95	96
1321	85	86	84	**2185**	92	94	94	**F2170-17**	87	87	85	**G-028**	93	91	91
1370	64	62	59	**2226**	72	73	71	**F2175-17**	77	77	77	**G-029**	94	96	96
1371	83	82	81	**2234**	81	82	78	**F2176-17**	74	74	72	**G-030**	82	79	79
1372	80	76	80	**2237**	69	72	70	**EGDe**	92	89	96	**G-031**	91	92	90
1373	80	81	82	**2240**	90	87	90	**10403S**	74	73	74	**G-033**	91	91	90
1374	83	81	81	**2241**	86	87	88	**MQ130026**	84	83	82	**G-034**	96	97	93
1375	82	80	79	**2256**	92	91	90	**MQ130029**	93	91	90	**G-035**	97	99	98
1376	80	80	80	**2259**	76	78	75	**MQ130032**	90	88	91	**G-036**	97	96	98
1377	87	86	85	**2294**	78	78	80	**MQ130033**	90	91	91	**G-037**	91	90	88
1378	72	76	73	**2298**	91	92	91	**MQ130037**	83	88	88	**G-038**	101	97	98

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
	Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3		Result 1	Result 2	Result 3
1379	88	85	88	**2507**	95	99	99	**MQ130042**	98	99	98	**G-039**	99	98	100
1380	86	84	85	**3442**	84	81	80	**MQ130058**	91	89	86	**G-040**	88	89	87
1381	89	88	89	**3443**	96	96	97	**MQ140011**	88	90	89	**G-041**	73	74	70
1382	82	82	83	**3444**	87	88	87	**MQ140012**	90	92	91	**G-042**	85	88	88
1383	83	82	85	**3445**	80	82	80	**MQ140025**	100	97	99	**G-043**	93	93	92
1384	76	79	80	**3446**	90	90	91	**MQ140029**	91	88	90	**G-044**	92	98	95
1385	87	86	84	**3447**	80	79	82	**MQ140030**	87	88	88	**G-045**	99	92	96
1386	89	92	87	**3448**	96	96	99	**MQ140031**	81	84	84	**G-046**	91	91	92
1387	77	77	76	**6179**	99	101	99	**MQ140032**	88	87	88	**G-047**	90	93	93
1388	52	56	56	**1513C**	77	80	77	**MQ140033**	91	89	87	**G-048**	94	98	95

Bile Resistance Assay – Aerobic Results

Table 3. **Aerobic** Bile Resistance Assay Results. Difference between Time 0 and Time 1 values were used to calculate bile resistance. Samples were tested in biological and technical triplicates.

Sample ID	**% Bile Resistance**			**Sample ID**	**% Bile Resistance**			**Sample ID**	**% Bile Resistance**			**Sample ID**	**% Bile Resistance**		
	Result 1	**Result 2**	**Result 3**		**Result 1**	**Result 2**	**Result 3**		**Result 1**	**Result 2**	**Result 3**		**Result 1**	**Result 2**	**Result 3**
946	95	99	96	**1798**	97	99	100	**MQ140011**	97	102	99	**G-016**	97	95	98
948	100	99	102	**1976**	96	98	99	**MQ140012**	99	100	100	**G-017**	95	99	98
950	101	98	104	**1991**	97	99	99	**MQ140025**	96	99	99	**G-018**	97	99	96
954	103	97	97	**2105**	98	99	94	**MQ140029**	98	98	99	**G-019**	102	99	99
966	98	97	97	**2113**	95	93	99	**MQ140030**	98	96	96	**G-020**	98	94	99
976	101	99	101	**2181**	99	95	100	**MQ140031**	93	94	92	**G-021**	96	95	97

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
987	97	96	97	**2183**	95	99	98	**MQ140032**	99	99	99	**G-022**	95	94	95
991	102	98	100	**2185**	103	98	100	**MQ140033**	100	99	100	**G-023**	99	100	99
997	101	100	102	**2234**	94	95	96	**MQ140034**	98	100	97	**G-024**	95	97	96
1006	97	97	98	**2240**	96	95	100	**MQ140035**	101	99	99	**G-025**	97	99	98
1013	101	100	97	**2241**	96	98	98	**MQ150001**	97	102	99	**G-026**	96	97	97
1019	99	98	100	**2256**	99	98	98	**MQ150004**	98	99	97	**G-027**	99	100	98
1021	95	97	97	**2294**	99	99	96	**MQ150005**	99	101	97	**G-028**	98	100	98
1147	99	100	97	**2298**	95	97	100	**MQ150007**	101	100	99	**G-029**	101	97	99
1155	98	101	97	**6179**	98	99	99	**MQ150008**	98	101	98	**G-030**	92	94	94

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
1268	98	99	96	**F2365**	94	95	96	**MQ150011**	100	98	101	**G-031**	95	97	96
1304	99	98	98	**F347-16**	98	98	99	**MQ150012**	98	99	99	**G-033**	98	98	98
1306	99	97	99	**F1209-16**	98	100	96	**MQ150013**	97	98	101	**G-034**	98	95	99
1313	99	101	99	**F1221-16**	101	100	98	**G-001**	100	97	99	**G-035**	99	98	102
1321	97	99	101	**F112-17**	96	102	99	**G-002**	102	98	100	**G-036**	93	92	92
1383	101	98	98	**F1099-17**	101	98	97	**G-003**	103	100	100	**G-037**	98	99	96
1386	99	97	96	**F1646-17**	99	101	95	**G-004**	96	98	99	**G-038**	97	99	97
1403	96	99	99	**F2152-17**	99	96	96	**G-005**	98	98	96	**G-039**	96	97	95
1423	96	99	97	**F2154-17**	99	98	97	**G-006**	100	95	97	**G-040**	97	102	103

Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance			Sample ID	% Bile Resistance		
1428	97	99	95	**F2166-17**	97	97	99	**G-007**	95	96	99	**G-042**	101	95	98
1441	93	95	96	**F2170-17**	95	91	90	**G-008**	95	96	101	**G-043**	95	99	97
1507	99	99	95	**EGDe**	97	99	95	**G-009**	98	96	98	**G-044**	97	98	95
1515	100	100	101	**MQ130026**	97	98	95	**G-010**	98	98	100	**G-045**	102	95	97
1527	98	97	95	**MQ130033**	99	101	99	**G-011**	91	92	90	**G-046**	95	100	97
1534	96	97	99	**MQ130037**	99	100	100	**G-012**	96	96	98	**G-047**	100	99	98
1564	96	97	97	**MQ130042**	96	96	95	**G-013**	94	98	96	**G-048**	98	99	97
1679	99	99	99	**MQ130058**	98	97	95	**G-014**	99	100	100				

Bile Salt Hydrolase-Agar Plate Assay Results

Table 4. Bile Salt Hydrolase Agar Plate Results for *L. monocytogenes* cultures patch inoculated onto 0.5% GDCA and TDCA MRS agar. BSH zone size diameters were measured after 48- and 72-hours anaerobic incubation. Samples were tested in biological and technical duplicates.

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				**0.5% GDCA MRS Agar**						**0.5% TDCA MRS Agar**					
Sample ID	**Category**	**Date Tested**	**Bile Agar Batch**	**48 Hour Diameter (mm)**		**Score**	**72 Hour Diameter (mm)**		**Score**	**48 Hour Diameter (mm)**		**Score**	**72 Hour Diameter (mm)**		**Score**
EGDe	Control	12/05/2021	18/04/2021	8		+++	8		+++	0		-	3		+
EGDe	Control	22/05/2021	09/05/2021	5.5		++	6		++	3		+	3		+
EGDe	Control	02/06/2021	30/05/2021	6		++	9.5		+++	4		++	5		++
EGDe	Control	03/10/2021	30/05/2021	10		++++	14		++++	4		++	4		++
EGDe	Control	14/06/2022	12/06/2022	8		+++	9		+++	5		++	6		++
L. innocua	Control	25/04/2021	18/04/2021	0		-	0		-	0		-	0		-
944	Food/Env	03/10/2021	30/05/2021	6	6	++	8	8	+++	0	0	-	4	4	++
946	Food/Env	02/06/2021	30/05/2021	4	5	++	5	5	++	0	0	-	0	0	-
948	Food/Env	02/06/2021	30/05/2021	5	4	++	8	8	+++	0	0	-	0	0	-
950	Food/Env	02/06/2021	30/05/2021	6	8	++/+++	9	9	+++	0	0	-	0	0	-
954	Food/Env	20/06/2021	30/05/2021	5	7	++/+++	6	7	++/+++	0	0	-	0	0	-
958	Food/Env	12/05/2021	18/04/2021	4	4	++	5	5	++	0	0	-	0	0	-
960	Food/Env	03/10/2021	30/05/2021	7	7	+++	9	9	+++	0	0	-	0	0	-
965	Food/Env	12/05/2021	18/04/2021	5	5	++	6	6	++	0	0	-	0	0	-
966	Food/Env	02/06/2021	30/05/2021	5	7	++/+++	6	7	++/+++	0	0	-	0	0	-

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
970	Food/Env	16/05/2021	09/05/2021	8.5	8.5	+++	10	10	++++	0	0	-	0	0	-
974	Food/Env	12/05/2021	18/04/2021	8	8	+++	11	11	++++	0	0	-	2.5	2.5	+
976	Food/Env	02/06/2021	30/05/2021	4	7	++/+++	8	8	+++	0	0	-	0	0	-
987	Food/Env	02/06/2021	30/05/2021	7	8	+++	9	9	+++	0	0	-	0	0	-
991	Food/Env	02/06/2021	30/05/2021	6	5	++	7	7	+++	3.5	2	+	3.5	3.5	+
997	Food/Env	02/06/2021	30/05/2021	6	8	++/+++	8	8	+++	4	5	++	4	5	++
1003	Food/Env	12/05/2021	18/04/2021	8	8	+++	9	9	+++	0	0	-	2.5	2.5	+
1006	Food/Env	02/06/2021	30/05/2021	6	6	++	8	8	+++	0	0	-	0	0	-
1013	Food/Env	02/06/2021	30/05/2021	5	8	++/+++	6	8	++/+++	0	0	-	0	0	-
1019	Food/Env	20/06/2021	30/05/2021	5	6	++	5	6	++	3.5	2.5	+	3.5	3.5	+
1021	Food/Env	02/06/2021	30/05/2021	5.5	5	++	6	6	++	0	0	-	0	0	-
1023	Food/Env	02/06/2021	30/05/2021	6	6	++	6	6	++	3	3	+	3	3	+
1055	Food/Env	12/05/2021	09/05/2021	4.5	4.5	++	6	6	++	0	0	-	0	0	-
1093	Food/Env	12/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	0	0	-
1095	Food/Env	20/06/2021	30/05/2021	8	6	+++/++	8	8	+++	3	2	+	3	3	+
1101	Food/Env	03/10/2021	30/05/2021	6	6	++	10	10	++++	0	0	-	0	0	-
1107	Food/Env	12/05/2021	09/05/2021	3.5	3.5	+	6	6	++	0	0	-	2.5	2.5	+
1109	Food/Env	12/05/2021	09/05/2021	6	6	++	8	8	+++	0	0	-	0	0	-
1123	Food/Env	12/05/2021	09/05/2021	8	8	+++	11	11	++++	0	0	-	2.5	2.5	+
1147	Food/Env	20/06/2021	30/05/2021	6	4	++	6	6	++	3	2.5	+	3	3	+
1155	Food/Env	02/06/2021	30/05/2021	5.5	5	++	7.5	6	+++/++	4	0	++/-	4.5	0	++/-

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
1203	Food/Env	02/06/2021	30/05/2021	8	9	+++	12	12	++++	0	0	-	0	0	-
1268	Food/Env	02/06/2021	30/05/2021	5	7	++/+++	6	8	++/+++	0	0	-	0	0	-
1304	Food/Env	20/06/2021	30/05/2021	6	7	++/+++	7	7	+++	3.5	2.5	+	3.5	3.5	+
1306	Food/Env	02/06/2021	30/05/2021	6	6	++	7	7	+++	0	0	-	0	0	-
1309	Food/Env	12/05/2021	09/05/2021	8	8	+++	10	10	++++	0	0	-	0	0	-
1313	Food/Env	02/06/2021	30/05/2021	6	6	++	6	7	++/+++	5	0	++/-	5	5	++
1317	Food/Env	16/05/2021	09/05/2021	8	8	+++	8	8	+++	0	0	-	0	0	-
1321	Food/Env	20/06/2021	30/05/2021	7	6	+++/++	8	8	+++	3	2.5	+	3	3	+
1370	Food/Env	09/06/2021	30/05/2021	5	4	++	5	5	++	0	0	-	0	0	-
1371	Food/Env	09/06/2021	30/05/2021	7	7	+++	8	8	+++	4.5	4.5	++	5	5	++
1372	Food/Env	09/06/2021	30/05/2021	5	5	++	6	6	++	0	0	-	0	0	-
1373	Food/Env	09/06/2021	30/05/2021	9	5	+++/++	10	8	++++/+++	6	4	++	6	4	++
1374	Food/Env	09/06/2021	30/05/2021	5	5	++	6	6	++	0	0	-	0	0	-
1375	Food/Env	09/06/2021	30/05/2021	9	8	+++	9	9	+++	0	0	-	0	0	-
1376	Food/Env	09/06/2021	30/05/2021	5	6	++	5	6	++	0	0	-	0	0	-
1377	Food/Env	09/06/2021	30/05/2021	8	6	+++/++	8	8	+++	3	2	+	3	3	+
1378	Food/Env	09/06/2021	30/05/2021	4	4	++	4	4	++	0	0	-	0	0	-
1379	Food/Env	09/06/2021	30/05/2021	4	5	++	6	6	++	0	0	-	0	0	-
1380	Food/Env	09/06/2021	30/05/2021	6	4	++	7	7	+++	5	2	++/+	5	5	++
1381	Food/Env	09/06/2021	30/05/2021	6	7	++/+++	6	7	++/+++	3	3	+	3	3	+
1382	Food/Env	09/06/2021	30/05/2021	5	6	++	6	6	++	4	2.5	++/+	4	4	++

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
1383	Food/Env	02/06/2021	30/05/2021	6	5	++	8	8	+++	0	0	-	0	0	-
1384	Food/Env	09/06/2021	30/05/2021	8	5	+++/++	9	9	+++	0	0	-	0	0	-
1385	Food/Env	09/06/2021	30/05/2021	6	7	++/+++	9	9	+++	3.5	3.5	+	3.5	3.5	+
1386	Food/Env	20/06/2021	30/05/2021	7	6	+++/++	8	8	+++	3	2	+	3	3	+
1387	Food/Env	09/06/2021	30/05/2021	6	4	++	8	8	+++	3.5	3	+	3.5	3.5	+
1388	Food/Env	09/06/2021	30/05/2021	0	0	-	0	0	-	0	0	-	0	0	-
1389	Food/Env	09/06/2021	30/05/2021	8	7	+++	9	9	+++	2.5	2	+	2.5	2.5	+
1390	Food/Env	09/06/2021	30/05/2021	6	5	++	8	8	+++	4	3	++	4	4	++
1391	Food/Env	09/06/2021	30/05/2021	4	3	++/+	5	5	++	0	0	-	0	0	-
1392	Food/Env	02/06/2021	30/05/2021	6	8	++/+++	7	8	+++	0	0	-	0	0	-
1393	Food/Env	09/06/2021	30/05/2021	8	6	+++/++	8	7	+++	3	3	+	3	3	+
1394	Food/Env	09/06/2021	30/05/2021	7	6	+++/++	9	9	+++	3	3.5	+	3	3.5	+
1403	Food/Env	02/06/2021	30/05/2021	7	6	+++/++	7.5	7.5	+++	0	0	-	0	0	-
1411	Food/Env	12/05/2021	09/05/2021	7	7	+++	8.5	8.5	+++	0	0	-	3	3	+
1413	Food/Env	02/06/2021	30/05/2021	5	4	++	5	5	++	0	0	-	0	0	-
1423	Food/Env	02/06/2021	30/05/2021	8	8	+++	8	8	+++	3.5	4	+/++	3.5	4	+/++
1427	Food/Env	12/05/2021	09/05/2021	4.5	4.5	++	6	6	++	0	0	-	0	0	-
1428	Food/Env	20/06/2021	30/05/2021	6	6	++	8	8	+++	0	0	-	0	0	-
1439	Food/Env	12/05/2021	09/05/2021	7	7	+++	10	10	++++	0	0	-	0	0	-
1441	Food/Env	20/06/2021	30/05/2021	6	4	++	6	5	++	0	0	-	0	0	-
1445	Food/Env	16/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	0	0	-

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
1502	Food/Env	12/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	2.5	2.5	+
1507	Food/Env	02/06/2021	30/05/2021	7	5	+++/++	11	11	++++	4	2.5	++/+	4	4	++
1513	Food/Env	12/05/2021	09/05/2021	7	7	+++	9	10	+++/++++	0	0	-	0	0	-
1515	Food/Env	20/06/2021	30/05/2021	6	4.5	++	10	10	++++	4	2	++/+	4	4	++
1519	Food/Env	16/05/2021	09/05/2021	8	8	+++	9	9	+++	0	0	-	0	0	-
1527	Food/Env	02/06/2021	30/05/2021	6	5	++	6	6	++	0	0	-	0	0	-
1534	Food/Env	28/04/2021	18/04/2021	7.5	7.5	+++	N/T	N/T	N/T	2.5	2.5	+	N/T	N/T	N/T
1564	Food/Env	20/06/2021	30/05/2021	6	7	++/+++	7	7	+++	3	3	+	3	3	+
1673	Food/Env	09/06/2021	30/05/2021	8	8	+++	9	9	+++	0	4	-/++	0	4	-/++
1679	Food/Env	20/06/2021	30/05/2021	6	8	++/+++	7	8	+++	3	4	+/++	4	4	++
1734	Food/Env	12/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	2.5	2.5	+
1798	Food/Env	12/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
1880	Food/Env	12/05/2021	09/05/2021	4.5	4.5	++	6	6	++	0	0	-	0	0	-
1976	Food/Env	20/06/2021	30/05/2021	6	6.5	++	6	6.5	+	0	0	-	0	0	-
1989	Food/Env	12/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	2	2	+
1991	Food/Env	20/06/2021	30/05/2021	8	7	+++	10	10	++++	4	3	++/+	5	5	++
2075	Food/Env	12/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	5.5	5.5	++
2095	Food/Env	16/05/2021	09/05/2021	4.5	4.5	++	4.5	4.5	++	0	0	-	0	0	-
2105	Food/Env	20/06/2021	30/05/2021	5	6	++	6	6	++	3	2	+	3	3	+
2113	Food/Env	02/06/2021	30/05/2021	7.5	9	+++	10	10	++++	4	6	++	4	6	++
2181	Food/Env	20/06/2021	30/05/2021	6	6	++	6	6	++	0	0	-	0	0	-

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
2183	Food/Env	02/06/2021	30/05/2021	6	5	++	6	6	++	3	3	+	3	3	+
2185	Food/Env	20/06/2021	30/05/2021	7	7	+++	8	8	+++	3	2.5	+	3	3	+
2226	Food/Env	16/05/2021	09/05/2021	5.5	5.5	++	5.5	5.5	++	0	0	-	0	0	-
2234	Food/Env	20/06/2021	30/05/2021	6	4	++	6	6	++	0	0	-	0	0	-
2237	Food/Env	12/05/2021	09/05/2021	4	4	++	5.5	5.5	++	0	0	-	2	2	+
2240	Food/Env	02/06/2021	30/05/2021	6	7	++/+++	8	8	+++	3.5	3	+	3.5	3.5	+
2241	Food/Env	20/06/2021	30/05/2021	8	8	+++	8	8	+++	4.5	2.5	++/+	5	5	++
2256	Food/Env	02/06/2021	30/05/2021	7	6	+++/++	8	8	+++	5	2	++/+	5	5	++
2259	Food/Env	16/05/2021	09/05/2021	5.5	5.5	++	6	6	++	0	0	-	0	0	-
2294	Food/Env	02/06/2021	30/05/2021	8	7	+++	9	9	+++	0	0	-	0	0	-
2298	Food/Env	02/06/2021	30/05/2021	7	5	+++/++	8	8	+++	3	2.5	+	3	3	+
2507	Food/Env	12/05/2021	09/05/2021	3.5	6	+/++	6	6	++	0	0	-	2	2	+
3442	Food/Env	16/05/2021	09/05/2021	8	8	+++	8	8	+++	0	0	-	0	0	-
3443	Food/Env	16/05/2021	09/05/2021	8	8	+++	8	8	+++	4.5	4.5	++	4.5	4.5	++
3444	Food/Env	16/05/2021	09/05/2021	8	8	+++	8	8	+++	6	6	++	7	7	+++
3445	Food/Env	16/05/2021	09/05/2021	4	4	++	4	4	++	0	0	-	0	0	-
3446	Food/Env	16/05/2021	09/05/2021	4	4	++	5	5	++	0	0	-	0	0	-
3447	Food/Env	16/05/2021	09/05/2021	6	6	++	7	7	+++	4	4	++	6	6	++
3448	Food/Env	16/05/2021	09/05/2021	6.5	5.5	++	8	8	+++	4.5	4.5	++	5	5	++
6179	Food/Env	15/06/2022	12/06/2022	4	4	++	5	5	++	0	0	-	0	0	-
10403S	Clinical	12/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	3.5	3.5	+

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
1513C	Food/Env	14/06/2022	12/06/2022	8	8	+++	9	9	+++	5	5	++	5	5	++
F2365	Food/Env	15/06/2022	12/06/2022	7	7	+++	8	8	+++	4	4	++	4	4	++
F1857-15	Food/Env	20/06/2021	30/05/2021	6	5	++	6	6	++	0	0	-	0	0	-
F1994-15	Food/Env	20/06/2021	30/05/2021	6	5.5	++	8	8	+++	0	0	-	0	0	-
F2299-15	Food/Env	20/06/2021	30/05/2021	6	5.5	++	6	6	++	0	0	-	0	0	-
F2442-15	Food/Env	20/06/2021	30/05/2021	5	4	++	5	5	++	0	0	-	0	0	-
F2446-15	Food/Env	20/06/2021	30/05/2021	8	8	+++	9	9	+++	4	4	++	4	4	++
F347-16	Food/Env	20/06/2021	30/05/2021	5	4	++	6	6	++	0	0	-	0	0	-
F365-16	Food/Env	20/06/2021	30/05/2021	8	8.5	+++	9	9	+++	3.5	5	+/++	3.5	5	+/++
F671-16	Food/Env	12/05/2021	09/05/2021	3.5	3.5	+	3.5	3.5	+	0	0	-	4	4	++
F991-16	Food/Env	09/06/2021	30/05/2021	4.5	3	++/+	5	5	++	0	0	-	0	0	-
F1209-16	Food/Env	12/05/2021	09/05/2021	5.5	5.5	++	6	6	++	0	0	-	0	0	-
F1221-16	Food/Env	28/04/2021	18/04/2021	7.5	7.5	+++	N/T	N/T	N/T	4.5	4.5	++	N/A	N/T	N/T
F1230-16	Food/Env	09/06/2021	30/05/2021	9	6	+++/++	9	8	+++	4.5	0	++/-	6	0	++/-
F1524-16	Food/Env	09/06/2021	30/05/2021	6	4.5	++	6	6	++	0	0	-	0	0	-
F111-17	Food/Env	12/05/2021	09/05/2021	6	6	++	8	8	+++	0	0	-	2.5	2.5	+
F112-17	Food/Env	02/06/2021	30/05/2021	7	8	+++	8	8	+++	5	5	++	5	5	++
F113-17	Food/Env	12/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	2	2	+
F114-17	Food/Env	09/06/2021	30/05/2021	7	6	+++/++	9	9	+++	4	3.5	++/+	4	4	++
F168-17	Food/Env	12/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	0	0	-
F340-17	Food/Env	09/06/2021	30/05/2021	6	5.5	++	8	8	++	4	2	++/+	5	5	++

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
F345-17	Food/Env	20/06/2021	30/05/2021	8	8.5	+++	10	10	++++	4	4.5	++	5	5	++
F347-17	Food/Env	20/06/2021	30/05/2021	8	10	+++/++++	8	10	+++/++++	4	8	++/+++	5	8	++/+++
F348-17	Food/Env	09/06/2021	30/05/2021	8	8	+++	10	10	++++	0	0	-	0	0	-
F742-17	Food/Env	20/06/2021	30/05/2021	5	5.5	++	6	6	++	3	2	+	3	3	+
F1099-17	Food/Env	20/06/2021	30/05/2021	8	7	+++	10	8	++++/+++	5	4	++	5	4	++
F1109-17	Food/Env	09/06/2021	30/05/2021	7	7	+++	8	8	+++	3	4	+/++	3	4	+/++
F1644-17	Food/Env	09/06/2021	30/05/2021	6	4.5	++	6	6	++	0	0	-	0	0	-
F1646-17	Food/Env	20/06/2021	30/05/2021	7	7	+++	9	9	+++	5	2.5	++/+	6	6	++
F2151-17	Food/Env	09/06/2021	30/05/2021	8	8	+++	10	10	++++	0	5	-/++	0	5	-/++
F2152-17	Food/Env	12/05/2021	09/05/2021	6	6	++	9	9	+++	0	0	-	0	0	-
F2153-17	Food/Env	12/05/2021	09/05/2021	7	7	+++	9	9	+++	0	0	-	3	3	+
F2154-17	Food/Env	12/05/2021	09/05/2021	8	8	+++	10	10	++++	0	0	-	0	0	-
F2155-17	Food/Env	09/06/2021	30/05/2021	8	8	+++	9	9	+++	4	5	++	4	5	++
F2160-17	Food/Env	09/06/2021	30/05/2021	8	8	+++	8	8	+++	4	3.5	++/+	4	4	++
F2161-17	Food/Env	09/06/2021	30/05/2021	9	7	+++	11	8	++++/+++	3	5	+/++	3	6	+/++
F2165-17	Food/Env	09/06/2021	30/05/2021	6	8	++/+++	6	9	++/+++	4	4	++	4.5	6	++
F2166-17	Food/Env	02/06/2021	30/05/2021	5	4	++	5	5	++	0	0	-	0	0	-
F2170-17	Food/Env	20/06/2021	30/05/2021	8	7	+++	8	8	+++	4	4	++	6	6	++
F2175-17	Food/Env	20/06/2021	30/05/2021	6	7	++/+++	6	7	++/+++	3.5	2	+	3.5	3.5	+
F2176-17	Food/Env	12/05/2021	09/05/2021	4	4	++	4	4	++	0	0	-	0	0	-
G-001	Clinical	16/05/2021	09/05/2021	8	8	+++	9	9	+++	0	0	-	0	0	-

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
G-002	Clinical	16/05/2021	09/05/2021	8	8	+++	9	9	+++	0	0	-	0	0	-
G-003	Clinical	16/05/2021	09/05/2021	8	8	+++	8	8	+++	0	0	-	0	0	-
G-004	Clinical	16/05/2021	09/05/2021	6	6	++	7	7	+++	2	2	+	4	4	++
G-005	Clinical	16/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
G-006	Clinical	16/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
G-007	Clinical	16/05/2021	09/05/2021	8	8	+++	10	10	++++	0	0	-	2	2	+
G-008	Clinical	16/05/2021	09/05/2021	8	8	+++	10	10	++++	2	2	+	5	5	++
G-009	Clinical	16/05/2021	09/05/2021	8	8	+++	10	10	++++	2	2	+	5	5	++
G-010	Clinical	16/05/2021	09/05/2021	5	5	++	7	7	+++	2	2	+	2	2	+
G-011	Clinical	16/05/2021	09/05/2021	9	9	+++	10	10	++++	4	4	++	4	4	++
G-012	Clinical	16/05/2021	09/05/2021	8	8	+++	9	9	+++	0	0	-	0	0	-
G-013	Clinical	16/05/2021	09/05/2021	4	4	++	6	6	++	0	0	-	0	0	-
G-014	Clinical	16/05/2021	09/05/2021	6.5	6.5	++	7	7	+++	0	0	-	0	0	-
G-015	Clinical	16/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	0	0	-
G-016	Clinical	16/05/2021	09/05/2021	5	5	++	7	7	+++	0	0	-	0	0	-
G-017	Clinical	16/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
G-018	Clinical	16/05/2021	09/05/2021	4	4	++	4	4	++	0	0	-	0	0	-
G-019	Clinical	16/05/2021	09/05/2021	4	4	++	4	4	++	0	0	-	0	0	-
G-020	Clinical	16/05/2021	09/05/2021	4.5	4.5	++	6	6	++	0	0	-	0	0	-
G-021	Clinical	16/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	0	0	-
G-022	Clinical	16/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	4.5	4.5	++

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
G-023	Clinical	19/05/2021	09/05/2021	3.5	3.5	+	5	5	++	0	0	-	0	0	-
G-024	Clinical	19/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
G-025	Clinical	19/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	3	3	+
G-026	Clinical	19/05/2021	09/05/2021	6	6	++	8	8	+++	2.5	2.5	+	4	4	++
G-027	Clinical	19/05/2021	09/05/2021	8	8	+++	8	8	+++	2.5	2.5	+	2.5	2.5	+
G-028	Clinical	19/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	3.5	3.5	+
G-029	Clinical	19/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	3	3	+
G-030	Clinical	19/05/2021	09/05/2021	8	8	+++	8	8	+++	0	0	-	3	3	+
G-031	Clinical	19/05/2021	09/05/2021	8	8	+++	9	9	+++	0	0	-	2.5	2.5	+
G-033	Clinical	19/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	0	0	-
G-034	Clinical	19/05/2021	09/05/2021	4.5	4.5	++	5	5	++	0	0	-	0	0	-
G-035	Clinical	19/05/2021	09/05/2021	6	6	++	8	8	+++	0	0	-	3	3	+
G-036	Clinical	19/05/2021	09/05/2021	0	0	-	0	0	-	0	0	-	0	0	-
G-037	Clinical	19/05/2021	09/05/2021	8	8	+++	8	8	+++	0	0	-	4	4	++
G-038	Clinical	19/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	0	0	-
G-039	Clinical	19/05/2021	09/05/2021	5	5	++	8	8	+++	0	0	-	3	3	+
G-040	Clinical	14/06/2022	12/06/2022	0	0	-	0	0	-	0	0	-	0	0	-
G-041	Clinical	19/05/2021	09/05/2021	7	6	+++/++	9	8	+++	0	5	-/++	3.5	5	+/++
G-042	Clinical	19/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	0	0	-
G-043	Clinical	19/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	3	3	+
G-044	Clinical	19/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	3	3	+

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
G-045	Clinical	19/05/2021	09/05/2021	6	6	++	8	8	+++	3	3	+	3	3	+
G-046	Clinical	19/05/2021	09/05/2021	8	8	+++	8	8	+++	0	0	-	4	4	++
G-047	Clinical	19/05/2021	09/05/2021	4	4	++	6	6	++	0	0	-	0	0	-
G-048	Clinical	19/05/2021	09/05/2021	7	7	+++	7	7	+++	0	0	-	0	0	-
MQ130026	Clinical	19/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	0	0	-
MQ130029	Clinical	19/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	3	3	+
MQ130032	Clinical	19/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
MQ130033	Clinical	19/05/2021	09/05/2021	0	0	-	0	0	-	0	0	-	0	0	-
MQ130037	Clinical	19/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
MQ130042	Clinical	19/05/2021	09/05/2021	8	6	+++/++	9	7	+++	0	0	-	3	0	+/-
MQ130058	Clinical	19/05/2021	09/05/2021	7	7	+++	8	8	+++	0	0	-	3	3	+
MQ140011	Clinical	19/05/2021	09/05/2021	5	5	++	5	5	++	0	0	-	0	0	-
MQ140012	Clinical	19/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-
MQ140025	Clinical	19/05/2021	09/05/2021	7	4	+++/++	8	4	+++/++	3	0	+/-	3	0	+/-
MQ140029	Clinical	19/05/2021	09/05/2021	0	5	-/++	5	5	++	0	0	-	0	0	-
MQ140030	Clinical	22/05/2021	09/05/2021	5	6	++	6	6	++	0	0	-	0	0	-
MQ140031	Clinical	22/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	0	0	-
MQ140032	Clinical	22/05/2021	09/05/2021	3	5	+/++	4	8	++/+++	0	0	-	0	0	-
MQ140033	Clinical	22/05/2021	09/05/2021	6.5	6.5	++	7.5	7.5	+++	0	0	-	0	0	-
MQ140034	Clinical	22/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	0	0	-
MQ140035	Clinical	22/05/2021	09/05/2021	6	6	++	6	6	++	0	0	-	0	0	-

Scoring: + 1-3mm ++ 4-6mm +++ 7-9mm ++++ 10mm plus				0.5% GDCA MRS Agar						0.5% TDCA MRS Agar					
Sample ID	Category	Date Tested	Bile Agar Batch	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score	48 Hour Diameter (mm)		Score	72 Hour Diameter (mm)		Score
MQ150001	Clinical	22/05/2021	09/05/2021	4	4	++	6	6	++	0	0	-	0	0	-
MQ150004	Clinical	22/05/2021	09/05/2021	5	5	++	6	6	++	0	0	-	0	0	-
MQ150005	Clinical	22/05/2021	09/05/2021	6	6	++	7	7	+++	0	0	-	3	3	+
MQ150007	Clinical	22/05/2021	09/05/2021	4	8	++/+++	5	9	++/+++	0	4	-/++	3	4	+/++
MQ150008	Clinical	22/05/2021	09/05/2021	4	6	++	5	7	++	0	0	-	0	0	-
MQ150011	Clinical	22/05/2021	09/05/2021	0	0	-	0	0	-	0	0	-	0	0	-
MQ150012	Clinical	22/05/2021	09/05/2021	6	6	++	7	8	+++	3.5	3	+	3.5	3	+
MQ150013	Clinical	22/05/2021	09/05/2021	6.5	4	++	7	5	+++/++	0	0	-	0	0	-